THE ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITIES OF *CYCLOPIA* SPECIES AND ACTIVITY-GUIDED FRACTIONATION OF *C. INTERMEDIA*

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

The antioxidant and antimutagenic activities of the aqueous extracts of unfermented and fermented plant material of the commercially utilised *Cyclopia* species (honeybush tea), *Cyclopia intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides* were assessed. Scavenging of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$), reduction of a ferric-tripyridyltriazine complex and inhibition of Fe^{2+} induced lipid peroxidation in a model membrane system (rat liver microsomes) were used as measures of antioxidant activity. Antimutagenic activity against 2-acetylaminofluorene (2-AAF) with metabolic activation using tester strain TA98 was determined with the *Salmonella typhimurium* antimutagenicity (AMES) assay.

Fermentation of the plant material had a significant ($P < 0.05$) effect on the total water soluble solids and their phenolic content. Unfermented species yielded higher total soluble solids (27-39%), total polyphenol (27-33%), flavanol (0.7-2.4%) and flavonol + flavone (3.2-8.5%) contents than their fermented counterparts (16-35%, 16-22%, 0.1-0.6% and 2.6-7.6%, respectively). Antioxidant activity correlated with the total polyphenol ($r \geq 079$; $P < 0.0001$) and flavanol ($r = 0.53$; $P < 0.0001$) contents of the extracts. The flavonol + flavone content gave a negative correlation with antimutagenic activity ($r = -0.60$; $P < 0.0001$). The major phenolic compounds, mangiferin + isomangiferin and hesperidin, decreased substantially with fermentation. Mangiferin was the most effective $ABTS^{\bullet+}$ scavenger and ferric reducer, but gave moderate inhibition of lipid peroxidation. Hesperidin, naringenin, eriodictyol and formononetin exhibited promutagenic activity.

Overall, unfermented *C. intermedia* and *C. sessiliflora* had the highest antioxidant activities, although *C. genistoides* contained significantly more mangiferin + isomangiferin (86% of the total polyphenol content as opposed to 39% for *C. sessiliflora*, 20% for *C. intermedia* and 12% for *C. subternata*). *Cyclopia genistoides* was the most potent of the fermented plant material in all the antioxidant assays. Fermentation decreased the antimutagenicity of the aqueous extracts of the most potent species, *C. intermedia* and *C. sessiliflora*, but it had no effect on the antimutagenicity of *C. subternata*. The latter, together with *C. sessiliflora*, were the most potent of the fermented species. *Cyclopia genistoides* changed from promutagenic to antimutagenic with fermentation. This is partly attributed to the decrease in its flavonol + flavone content.

The antioxidant and antimutagenic activities of unfermented *C. intermedia* were further investigated by subjecting it to extraction with solvents of different polarities (dichlorometane, ethyl acetate, methanol and water), followed by fractionation of the methanol extract by column chromatography on XAD and C_{18} of a less polar fraction recovered from XAD. The total polyphenol content of the solvent extracts and fractions correlated with their $ABTS^{\bullet+}$ and ferric reducing abilities ($r \geq 0.97$; $P < 0.05$). Inhibition of lipid peroxidation not only depended on phenolic content, but also on the polarity of the compounds, with the inhibitory effect increasing with the lipophilicity of the fractions, as found for the XAD fractions. The effect of polarity on antimutagenicity was also evident for the solvent extracts and XAD fractions. Ethyl acetate gave 87% inhibition of 2-AAF induced mutagenesis, compared to 9% by the sequential methanol extract. Removal of the ethyl acetate soluble compounds before methanol extraction resulted in a higher antimutagenic activity (32%), indicating a possible antagonistic effect of these compounds in the non-sequential methanol extract. The most polar fraction of the methanol extract, was promutagenic, while the other fractions were antimutagenic with, the least polar, having the highest antimutagenicity.

In all cases, whether for extracts, fractions or compounds, where promutagenicity was demonstrated, testing in the absence and presence of metabolic activation proved them not to be mutagenic.

UITTREKSEL

Water ekstrakte van ongefermenteerde en gefermenteerde plantmateriaal van kommersieel-benutte *Cyclopia* spesies, *C. intermedia*, *C. subternata*, *C. sessiliflora* en *C. genistoides*, is ten opsigte van antioksidant en antimutageniese aktiwiteite ge-evalueer. Blussing van die 2,2'-azino-di-(3-etielbensotiasolien-sulfoonaat) radikaal katioon ($ABTS^{\bullet+}$), reduksie van 'n Fe^{3+}- tripiridieltriasien kompleks en inhibisie van Fe^{2+}-geïnduseerde lipied peroksidasie in 'n model membraansisteem (rot lewer mikrosome) is gebruik as maatstawwe van antioksidant aktiwiteit. Antimutageniese aktiwiteit teen 2-asetielaminofluorien (2-AAF) met metaboliese aktivering in die toetsras TA98 is met die *Salmonella typhimurium* antimutagenisiteitstoets (AMES) bepaal.

Fermentasie van die plantmateriaal het 'n betekenisvolle ($P < 0.05$) effek op die totale water oplosbare vastestowwe en hul polifenolinhoud gehad. Die totale oplosbare vastestof (27-33%), flavanol (0.7-2.4%) en flavonol + flavoon (3.2-8.5%) inhoude van die ongefermenteerde spesies was hoër as die ooreenkomstige waardes (16-35%, 16-22%, 0.1-0.6% en 2.6-7.6%) vir die gefermenteerde spesies. Antioksidant aktiwiteit het met die totale polifenol ($r \geq 079$; $P < 0.0001$) en flavanol ($r = 0.53$; $P < 0.0001$) inhoude van die ekstrakte gekorreleer. Die flavonol + flavoon inhoud het 'n negatiewe korrelasie met antimutageniese aktiwiteit ($r = -0.60$; $P < 0.0001$) gehad. Die hoof fenoliese komponente, mangiferien + isomangiferien en hesperidien, het beduidend met fermentasie afgeneem. Mangiferien was die mees effektiewe $ABTS^{\bullet+}$ blusser and Fe^{3+} reduseerder, maar dit was minder effektief as inhibeerder van lipied peroksidasie. Hesperidien, naringenien, eriodiktiol and formononetien het promutageniese aktiwiteit getoon.

Algeheel het ongefermenteerde *C. intermedia* en *C. sessiliflora* die hoogste antioksidant aktiwiteite gehad, alhoewel *C. genistoides* beduidend meer mangiferien + isomangiferien bevat het (86% van die totale polifenol inhoud in teenstelling met 39% vir *C. sessiliflora*, 20% vir *C. intermedia* en 12% vir *C. subternata*). *Cyclopia genistoides* was die aktiefste van die gefermenteerde plantmateriaal in al die antioksidant toetse. Fermentasie het die antimutagenisiteit van die water ekstrakte van die mees potente spesies, *C. intermedia* en *C. sessiliflora*, verminder, maar dit het geen effek op die antimutagenisiteit van *C. subternata* gehad nie. Laasgenoemde, saam met *C. sessiliflora*, was die mees potente van die gefermenteerde spesies. Die aktiwiteit van *C. genistoides* het van promutagenies na

antimutagenies verander met fermentasie. Dit word deels toegeskryf aan die afname in die flavonol + flavoon inhoud.

Die antioksidant en antimutageniese aktiwiteite van ongefermenteerde *C. intermedia* is verder ondersoek deur dit aan ekstraksie met oplosmiddels van verskillende polariteite (dichlorometaan, etielasetaat, metanol en water) te onderwerp, opgevolg met fraksionering van die metanol ekstrak deur middel van kolom chromatografie op XAD en C_{18} van 'n minder polêre fraksie herwin vanaf XAD. Die totale polifenolinhoud van die oplosmiddel ekstrakte en fraksies het met hul $ABTS^{\bullet+}$ blussende en Fe^{3+} reduserende vermoëns ($r \geq 0.97$; $P < 0.05$) gekorreleer. Inhibisie van lipied peroksidasie het nie net van fenoliese inhoud afgehang nie, maar ook van die polariteit van die verbindings. Die inhiberende effek het met lipofilisiteit van die fraksies toegeneem, soos gevind vir die XAD fraksies. Die effek van polariteit op antimutagenisiteit was ook duidelik vir die oplosmiddel ekstrakte en XAD fraksies. Etielasetaat het 87% inhibisie van 2-AAF geïnduseerde mutagenesis gegee, in vergelyking met 9% deur die opvolgende metanol ekstrak. Verwydering van die etielasetaat oplosbare stowwe voor metanol ekstraksie het tot hoër antimutageniese aktiwiteit (32%) gelei, wat op 'n moontlike antagonistiese effek van hierdie verbindings in die nie-opvolgende metanol ekstrak gedui het. Die mees polêre fraksie van die metanol ekstrak was promutagenies, terwyl die ander fraksies antimutagenies was. Die minste polêre fraksie het die hoogste antimutagenisiteit gehad.

In alle gevalle waar promutagenisiteit aangetoon is, ongeag of dit vir ekstrakte, fraksies of suiwer verbindings was, het toetsing in die afwesigheid en teenwoordigheid van metaboliese aktivering bewys dat hulle nie mutagenies was nie.

I would like to dedicate this thesis to my parents

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CONTENTS

The language and style of this thesis is in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters, has, therefore been unavoidable.

CHAPTER 1

INTRODUCTION

In developed countries cancer and cardiovascular disease are the major causes of mortality and the prevalence of these diseases has caused a change in the attitude of Western society. The general public has developed an irrational suspicion of the safety and desirability of synthetic chemicals resulting in many consumers preferring to treat themselves with phytopharmaceuticals or 'herbal' preparations (Chen *et al.*, 1983; Tanaka, 1997). Most Americans (93%) believe that some foods can have beneficial health benefits besides their basic nutritive value and can delay the onset or reduce the risk of serious chronic diseases (Archer, 2001). The sale of phytopharmaceuticals or 'herbal' preparations has increased about 10% each year in most first world countries (Houghton & Raman, 1998). In 1998 the nutraceutical market in the United States was estimated to be approximately three times larger that the pharmaceutical market and it was forecasted that by 2000 the global market for functional foods would be approximately $17 billion (De Felice, 1998; Kwak & Jukes, 2001). *Camellia sinensis* tea, said to be one of the most popular beverages and consumed by over two thirds of the world's population (Kuroda & Hara, 1999), has attracted significant attention in recent years because of reported health benefits, in particular as an antioxidant, but also as an anticarcinogenic and anti-arteriosclerotic agent (Wang *et al.*, 1989). These pharmacological effects of tea are generally believed to be due to the actions of the flavonoids (Halliwell & Gutteridge, 1989). Flavonoids have been recognised as important for general health and protection against various degenerative diseases such as cancer and arteriosclerosis (Cook & Samman, 1996; Pietta & Simonetti, 1999).

Honeybush tea, a traditional South African herbal beverage prepared from *Cyclopia* species endemic to the Cape fynbos region, is a relatively new entrant on the global herbal tea market. Studies on its *in vitro* antioxidant (Hubbe, 2000) and antimutagenic (Marnewick *et al.*, 2000) activities have indicated the potential of honeybush tea as a functional beverage or a nutraceutical ingredient. The recent registration of patents on processing techniques for unfermented *Cyclopia* (De Beer *et al.*, 2001) and extracts (De Beer & Joubert, 2002) is a direct result of the market potential of *Cyclopia* as a health promoting agent. In the past marketing of honeybush in South Africa was limited to the fermented product, formed through oxidative changes that result in browning of the leaves and the development of the characteristic honey-like flavour (Du Toit & Joubert, 1998). Marketing of "green" honeybush, the unfermented tea, took place for the first time in 2002 and currently its use for the preparation of an extract for the cosmeceutical market, based on its antioxidant properties, is under investigation. The market for honeybush tea has changed during the past few years from predominantly local to predominantly export (N. Coetzee, Coetzee & Coetzee

Distributors, personal communication, 2001). The export of honeybush tea is however, limited by the relatively small annual production of *c.* 120 tons of mostly *C. intermedia*, harvested from natural populations (N. Coetzee, Coetzee & Coetzee Distributors, personal communication, 2001). Large plantations of *C. genistoides* are in the process of being established.

Honeybush tea has a low tannin content (Terblanche, 1982) and contains no or traces of caffeine (Greenish, 1881), which makes it suitable for children and patients with digestive and heart problems (Van Wyk, 1997). Traditionally various positive health effects have been associated with honeybush tea. Beneficial effects on the urinary system have been noted and anecdotal evidence suggests that honeybush can increase appetite, prevent stomach ulcers, stimulate milk production in breast-feeding woman, treat colic in babies and be used as a cough syrup in cases of chronic tonsillitis and lung infection (Watt & Breyer-Brandwijk, 1932; Rood, 1994).

The phenolic content of fermented *Cyclopia* is *c.* 26% of the soluble solids of a cup of tea (Hubbe, 2000). The major compounds present in most of the *Cyclopia* species have been said to be mangiferin, hesperidin and isosakuranetin (De Nysschen *et al.*, 1996) and recently the compounds mangiferin and hesperidin were quantified and identified as the major compounds from unfermented *C. intermedia*, *C. sessiliflora* and *C. genistoides* (Joubert *et al.*, 2002). Up to date, qualitative analysis of only fermented *C. intermedia* (Ferreira *et al.*, 1998; Kamara, 1999) and unfermented *C. subternata* (Brand, 2002) has been carried out. The phenolic compounds isolated from these two species were shown to differ by as much as 80%. Fermented *C. intermedia* was found to contain phenolic compounds such as the xanthones mangiferin and isomangiferin, the flavanones hesperetin, hesperidin, naringenin and eriodictyol, the isoflavones formononetin, afrormosin, pseudobaptigen, fujikinetin and calycosin, the flavone luteolin, the coumestans medicagol, flemichapparin and sophoracoumestan (Ferreira *et al.*, 1998) and other kaempferol and flavone glycosides (Kamara, 1999). Unfermented *C. subternata* afforded compounds such as the flavones luteolin and scolymoside, the flavanones hesperidin, hesperetin, narirutin and eriocitrin, a flavan glucoside and a kaempferol glycoside, the isoflavone orobol and the flavanol epigallocatechin 3-*O*-gallate (Brand, 2002). No coumestans were isolated from unfermented *C. subternata*. As a result of this large difference in phenolic composition caution should be taken in assigning health benefits to *Cyclopia* as a whole since the different species may have distinctive biological properties. Evidence of this already exists, by the differences in *in vitro* antioxidant activity of *C. intermedia*, *C. subternata*, *C. sessiliflora*, *C. genistoides* and *C. maculata* (Hubbe, 2000). Furthermore, fermentation has been shown to decrease the antioxidant activity of these species (Hubbe, 2000). Similarly, a decrease in antimutagenic activity of *C. intermedia* exhibited against 2-acetylaminoflourene and aflatoxin B_1 occurred with fermentation (Marnewick *et al.*, 2000).

Some of the phenolic compounds present in *C. intermedia* have been demonstrated to exhibit certain biological properties. The xanthone, mangiferin, is well known for its cardiotonic, spasmolytic, diuretic, antidepressant and antimicrobial actions (Simova *et al*, 1986; Tahara and Ibrahim, 1995; Carroll *et al.*, 1998; Peres *et al.*, 2000). Luteolin and hesperetin have been shown to be both strong antimutagens (Choi *et al.*, 1994; Lien *et al.*, 1999) and to have antioxidant activity (Rice-Evans *et al.*, 1996). The antioxidant activity of luteolin was found to be equal to that of the endogenous antioxidant enzyme, superoxide dismutase (Hubbe, 2000), and half that of quercetin (Hubbe, 2000; Nakasugi, 2000). Inhibition of linoleic acid peroxidation by isomangiferin, eriodictyol and mangiferin has been demonstrated (Hubbe, 2000). Naringenin was found to be much more effective than the soy isoflavone, genistein, in its ability to inhibit proliferation of human breast cancer cells in culture (Carrol *et al.*, 1998). Isoflavones such as formononetin with phytoestrogenic activity may prove beneficial in postmenopausal woman with respect to the risk factors for heart disease, menopausal symptoms and osteoporosis and in the prevention of breast cancer (Cassidy *et al.*, 2000). Epigallocatechin 3-*O*-gallate present in *C. subternata* has been found to be responsible for the high bio-antimutagenic activity of green tea (*Camellia sinensis*) in comparison with other traditionally drunk teas (Kada *et al.*, 1985).

The present study will aim at extending the current knowledge on the bio-activities of the species of *Cyclopia,* namely, *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides* with potential for commercialisation. Species will be evaluated for antimutagenic activity, towards the metabolically activated mutagen 2-acetylaminofluorene, and antioxidant activity through scavenging of the synthetic 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical, reduction of the ferric-tripyridyltriazine complex and inhibition of lipid peroxidation in a biological membranous fraction using rat liver microsomes. In addition, this study will attempt to identify fractions of unfermented *C. intermedia* with potent antioxidant and/or antimutagenic activity through activity-guided solvent partitioning and chromatographic fractionation. Knowledge gained on the potency of these fractions will contribute towards the development of nutraceutical extracts or ingredients for the functional food market.

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CHAPTER 2

LITERATURE REVIEW

1. INTRODUCTION

In recent years interest in cancer chemoprevention has increased, with natural antimutagens and antitumor promoters being sought (Nakasugi & Komai, 1998). Oxidative stress is involved in the pathology of cancer as well as other diseases such as arteriosclerosis, malaria, rheumatoid arthritis and coronary heart disease and could even play a role in neurodegenerative diseases and the ageing process (Davies *et al.*, 1995; Moure *et al.*, 2001). Lipid peroxidation and DNA oxidation occur as a result of oxidative reactions with free radicals (products of the cell's defence mechanisms) and have been identified as the cause and consequence of arteriosclerosis and cardiovascular disease and cancer, respectively. Dietary antioxidants may prevent excessive oxidative reactions and thus reduce the chances of mutagenesis, cancer and cardiovascular disease (Moure *et al.*, 2001).

The flavonoid constituents of honeybush tea have demonstrated good antioxidant and antimutagenic activity in *in vitro* systems based on scavenging of either a stable free radical or more short lived reactive oxygen and nitrogen species and the inhibition of mutagenesis induced in mutated strains of *Salmonella typhimurium* (Simova *et al.*, 1986; Tahara & Ibrahim, 1995; Carroll *et al.*, 1998; Hubbe, 2000; Marnewick *et al.*, 2000; Peres *et al.*, 2000). Anecdotal evidence has suggested beneficial effects of honeybush tea for the treatment of arthritis and skin ailments such as psoriasis, as well as in providing relief from urinary and digestive problems (Watt & Breyer-Brandwijk, 1932; Rood, 1994).

The use of phenolic compounds derived from *Cyclopia* as nutraceutical ingredients requires information on their biological properties as well as their structure-activity relationship, bio-availability and the effects these compounds have on the prevention of degenerative diseases such as cancer and arteriosclerosis. These topics as well as issues regarding the regulation of nutraceutical products will be discussed.

2. FUNCTIONAL FOODS AND NUTRACEUTICALS

"Let food be thy medicine and medicine be thy food" - Hippocrates

The nutraceutical revolution began in the early 1980s, sparked by the calcium, fibre, and fish oil phenomena (Hasler, 1996). The actual or potential clinical benefits of these substances were supported by clinical studies published in distinguished medical journals and expert physicians, who began to educate their colleagues and consumers about these products via the mass media. This was followed by a continuous stream of publications on clinical studies defining the potential benefits of a growing range of products on a rapidly expanding array of specific disease processes (Hasler, 1996). The arrival at a name that could specifically describe these products evolved from the casually assigned name *designer foods* to the more specifically and carefully defined term *nutraceutical*. The phrase *designer foods* was coined in 1989 and it described foods that naturally contained or were enriched with non-nutritive, biologically active chemical components of plants (e.g. phytochemicals) that were effective in reducing cancer risk (Hasler, 1996). This was superseded by the term *functional foods*, defined as foods that encompass potentially healthful products, including, modified foods or food ingredients that may provide a health benefit beyond the traditional nutrients it contains (Hasler, 1996). Nearing the end of the decade Stephen L. DeFelice coined the term *nutraceutical* to give a much-needed identity and legitimacy to this area of nomenclature (DeFelice, 1998). He defined a nutraceutical as any substance that is a food or part of a food and provides medicinal or health benefits, including the prevention and/or treatment of disease.

Increasing scientific evidence is supporting the role of phytochemicals, as functional foods, in the prevention and treatment of at least four of the leading causes of death namely cancer, diabetes, cardiovascular disease and hypertension (Bloch & Thomson, 1997). With the current emphasis on cost effective health care and the importance of dietary changes to optimise health, consumers are becoming more aware of the role food plays in the prevention of certain types of diseases (Bloch & Thomson, 1997). The response by the food industry has been phenomenal with the development and marketing of nutrient rich food products now known as *nutraceuticals* at an increasing rate of 10% each year in most first world countries (Houghton & Raman, 1998).

The functional food and nutraceutical market

According to a recent survey done by the International Food Information Council, 93% of Americans believe that some foods can have health benefits besides the nutritive value and can delay the onset, or reduce the risk, of serious and chronic diseases (Archer, 2001). In 1998 the Foundation for Innovation in Medicine estimated the size of the U.S. nutraceutical market to be $250 billion, approximately three times larger than the U.S. pharmaceutical market (DeFelice, 1998). However, the present market is comprised mostly of potential nutraceuticals lacking clinical evidence to support their clinical promise. It is therefore, expected that with the increasing clinical research the present nutraceutical market will decrease and the true nutraceutical market will emerge. This could explain the difference in forecasts made by other authors on the size of this market. Kwak & Jukes (2001), for instance, estimated that the global market for functional foods was approximately $17 billion in 2000.

In South Africa the availability of products incorporating antioxidative or antimutagenic phytochemicals has been restricted to mainly nutritional supplements boasting the incorporation of compounds such as procyanidin, soy isoflavones and selenium. However, just recently there has been an addition to these products in the form of a supplement incorporating phenolic compounds from the well-known South African herbal beverage, rooibos. The positive reception of this product by the public creates opportunities for the development of honeybush herbal extracts for the nutraceutical market.

The safety assurance and risk assessment of functional foods and nutraceuticals

Food-based materials such as nutraceuticals (with potential health or medicinal properties) fall into a grey area between foods and medicines. The long-term use of traditional foods has given people experience in how to use them and how to manage potentially adverse effects, whereas this experience is lacking for nutraceuticals. Some form of safety assessment is required to identify the potential risks and this evaluation must address both nutritional and toxicological considerations (Huggett, 1996). Nutraceuticals should at least be as safe as their traditional counterparts (when these exist) or should not add significantly to the existing risks of dietary origin.

The notion of using traditional foods to assess the comparative safety of nutraceuticals was developed into the concept of "substantial equivalence" (Huggett, 1996). The concept can be applied to nutraceuticals if it can be demonstrated that the nutraceutical is substantially equivalent or sufficiently similar to the traditional, acceptable reference food. This may then

permit a suitable safety assessment with a reduced need for extensive toxicological and nutritional studies (Huggett, 1996). It is, however, important to keep in mind that it may be possible that extraction of substances could result in a situation where safety is in question. The safety of individual compounds or mixtures of compounds when present in a particular food does not ensure its safety once extracted from that food. For example, a compound or mixture of compounds could be removed from a protective substance or activated to an injurious form upon extraction from the original product (Clydesdale, 1996). Therefore it may be important that the necessary risk assessment be conducted through a combination of epidemiological studies, intervention trials and investigations into biological mechanisms, through chemical, cellular, or animal models that investigate plausible mechanisms of action (Clydesdale, 1996).

Regulations governing supplements, herbal remedies and functional foods

Food laws and regulations are enacted to protect the health of the consumer, to prevent economic fraud and to ensure the essential quality and wholesomeness of food. Laws are especially necessary for consumer protection regarding nutraceuticals and functional foods due to confusion and wilful distortion of claims for the health giving properties of these products by the producers (Hegarty, 2000). Since four fifths of all people in the world still rely to a great extent on traditional medicines based on plants and their components, systematic attempts are being made to codify the use and sale of these traditional medicines into acceptable regulations (Hegarty, 2000).

Regulations regarding the sale of nutraceuticals and functional foods differ greatly from country to country. The Codex Alimentarius, established in 1961 and linking 165 member countries of which South Africa is one, is attempting to unify these principles by developing fair international guidelines regarding food legislation (Pettman, 2002). There are currently four key Codex initiatives directly affecting the dietary supplements sector, with key implications for the functional foods sector: the draft guideline on vitamin and mineral supplements; the draft standard on additives, including those that may be used in supplements; the draft guideline for health claims that can be used for foods, including supplements; and the principles for assessing and managing risks (Pettman, 2002). In the following section a brief overview of the regulations governing functional foods and nutraceuticals, in countries such as Japan, the United Stated and Europe, that are at the forefront of regulatory control, will be discussed.

Japan

Japan is viewed as the world pioneers in the development of functional foods, in part perhaps for reasons linked to the Japanese culture and also because of the proactive stance the government has taken (Burke, 2000). In 1984 the Ministry of Education, Science and Culture in Japan sponsored a research project into functional foods and by 1989 the Functional Foods Liaison Board was established to serve as the sole intermediary with industry with individual subcommittees to deal with each potential food ingredient. The "Tokutei Hokenyo Shokuhin" or "foods for specified health use" (FOSHU) has become the official term given to these products outside Japan and only products with voluntary FOSHU approval may make health claims in Japan (Yamaguchi, 2001; Burke, 2000; Ichikawa, 1994). By May 2001 two hundred and fifty two products were permitted to carry the FOSHU logo and a specific health claim on the package label. A consumer study conducted by the industry trade organisation, Japan Health Food & Nutrition Food Association, showed that 65% of people aged 35-55 are aware of FOSHU products and 22% of those surveyed, reported using FOSHU products regularly (Yamaguchi, 2001).

United States

With regard to nutritional labelling in the United States the regulations, relating to nutrient content and nutrient content claims, were enacted in the Nutritional Labelling and Education Act of 1990 (Strobos, 1998). Health claims that are statements on the labelling of foods which "characterises the relationship" of a food substance to "a disease or health-related condition" are subject to prior authorisation by the FDA before they may be used on a food product. However, dietary supplements are permitted to make four types of statements without obtaining FDA approval as long as the label states that the statement has not been evaluated by the FDA and that the product is not intended to diagnose, treat, cure or prevent any disease (Giese & Katz, 1997). Statements that may be made without FDA approval are: a statement that claims a benefit related to a classic deficiency disease and states the prevalence of that disease among American public; a statement that describes the role of a nutrient or dietary ingredient intended to affect the structure or function in humans; a statement that discusses documented mechanisms by which a nutrient or dietary ingredient acts to maintain the structure or function; or a statement that describes the general well-being from consumption of a nutrient or dietary ingredient (Giese & Katz, 1997).

The future of the functional food industry in the United States will lie in the development of narrowly focussed products for specific market segments or disorders

(Gardner, 1994). In the USA half of the women over 45 years and 90% of women over 75 years of age are affected by osteoporosis, leading to at least 1.3 million fractures per year (Brouns & Vermeer, 2000). Potter *et al.* (1998) recently found that dietary supplementation with soy preparations rich in genistein significantly increased bone mineral density thus reducing the chances of osteoporosis. The average dietary isoflavone intake in Japan, the country with the highest soy consumption in the world (350±200 mg day^{-1}) and the lowest incidence of osteoporosis, is within the range proved to provide significant protection against the effects of osteoporosis. Based on this data it seems that there may be a market for phyto-estrogen enriched products in countries where the consumption of soy based foods historically is limited. Thus, plant estrogens may be promising candidates for functional foods against osteoporosis.

Europe

Most of the regulation governing food ingredients in Europe have been in place for decades and have received only minor revisions in subsequent years (Gardner, 1994; Hegarty, 2000). There is no clear indication that the European community will move to follow the United States in the development of new legislation focused on increasing consumer awareness and industry standardisation. The European community is more likely to continue to act to preserve consumer safety while not limiting inter-European trade. Most of the European community favours the development of food products that are based on natural ingredients proven to be safe (Gardner, 1994). While the European community and independent nations have not overtly reacted to the establishment and development of the functional food business, limitations are placed on the therapeutic claims that can be made (Hegarty, 2000). Such limitations may dictate the inclusion of a disclaimer on a product with a specific health claim. An example of such a disclaimer would be: *a traditional remedy for the systematic relief of... and if symptoms persist consult your doctor* (Hegarty, 2000; Ottaway, 2000).

3. ANTIOXIDANT COMPOUNDS IN DISEASE PREVENTION

An antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate

(Halliwell & Gutteridge, 1989)

In recent years there has been an increase in healthy lifestyles and healthy ageing with a corresponding increase of interest in antioxidants and food supplements. Few antioxidants are regarded as essential and include vitamins E and C and selenium. Vitamin E is perhaps the most well known of the nutrient antioxidants and was one of the first antioxidants for which epidemiological evidence indicated that health affects accrued when it was consumed in amounts greater than could be found in the diet (e.g. protection against heart disease) (Halliwell & Gutteridge, 1989). Other nutrient antioxidants, while not essential, are endogenous cellular components and are coming under increasing scrutiny as they offer numerous health benefits. Antioxidants from natural sources contain a bewildering array of compounds which represent a vast and still little studied area.

Antioxidants function by preventing oxidative reactions that would have otherwise damaged either the lipids in cell membranes, proteins, or the DNA, the basic instruction manual for the body's functions. This oxidative damage appears to be a major contributing factor to ageing and the many degenerative processes associated with it, including cancer, heart disease, cataracts, and cognitive dysfunction (Jovanovic *et al.*, 1998). A healthy cell should be capable of maintaining the balance between production and inactivation of free radicals such as the superoxide radical, however, during prolonged inflammation an overproduction of the superoxide radical occurs which leads to the formation of other more reactive species (Jovanovic *et al.*, 1998). If not inactivated by chemical or biochemical defences, this excess superoxide leads to oxidative stress resulting in degenerative diseases associated with this cell damage. Existence in an oxidative environment requires living organisms to generate or garner from their surroundings a variety of water and lipid soluble antioxidant compounds to aid the endogenous antioxidant enzymes. Thus, the working hypothesis on the beneficial effects of plant extracts in inflammatory diseases is that the excess superoxide and its reactive by-products are eliminated by flavonoids (Halliwell & Gutteridge, 1989).

Oxidative stress

The paradox of aerobic life, or the 'Oxygen Paradox', is that higher eukaryotic aerobic organisms cannot exist without oxygen, yet oxygen is inherently dangerous to their existence. Oxygen constitutes 20.9% of air and is paramagnetic, being a biradical in triplet ($^3\Sigma_g^-$) state with two electrons in separate antibonding orbitals (Namiki, 1990). Due to this peculiarity, oxygen molecules have weaker interatomic bonding than nitrogen molecules and can form complexes with oxygen as electron acceptor. Triplet oxygen can react with elements and ions to form oxides, but usually not with organic compounds that are in singlet states. It reacts easily with free radical molecules produced by the action of other active radicals, radiation, ultraviolet light, heat, or by complex formation with oxygen and transition metal ions to produce active peroxide radicals and trigger oxidation of unsaturated fatty acids. The reductive environment of the cellular milieu provides ample opportunity for oxygen to undergo unscheduled univalent reduction. Thus the superoxide anion radical, hydrogen peroxide and the extremely reactive hydroxyl radical are common products of life in an aerobic environment, and these agents appear to be responsible for oxygen toxicity (Davies, 1995). The activation of phagocytes by an inflammatory reaction causes degranulation of mast cells and the subsequent production of superoxide and hydrogen peroxide that is essential for the killing of bacteria (Halliwell, 1987). Although only mildly reactive towards biological molecules, the superoxide radical may be transformed to the more reactive and damaging hydroxyl radical in the Haber-Weiss and Fenton reactions. Oxidative reactions brought about by this radical may lead to degenerative diseases associated with lipid peroxidation and DNA oxidation such as coronary heart disease and arteriosclerosis or cancer and even the ageing process, respectively.

Lipid peroxidation

Lipids containing polyunsaturated fatty acids and their esters are readily oxidised by molecular oxygen (Wheatley, 2000). Such oxidation, called autoxidation, can be defined as the introduction of a functional group containing two catenated oxygen atoms, O-O, into unsaturated fatty acids in a free radical chain mechanism (Niki, 1987; Wheatley, 2000).

The chain reaction consists of three steps namely initiation, propagation and termination and is initiated by the formation of a carbon-centred radical through hydrogen abstraction from the lipid (Figure 1) (Niki, 1987; Wheatley, 2000). In the initiation step the

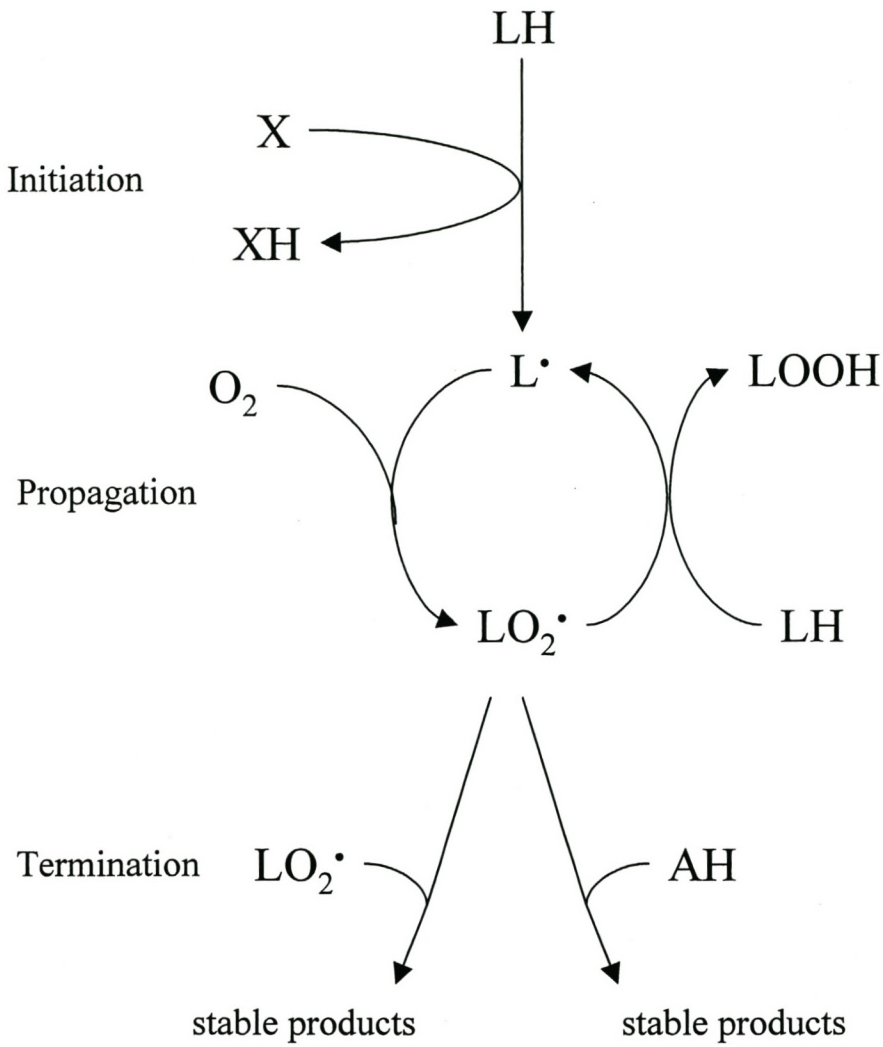


Figure 1. General scheme for the oxidation of lipids by a free radical chain mechanism (lipid (LH), lipid radical (L$^{\bullet}$), lipid peroxyl radical ($LO_2^{\bullet}$), antioxidant (AH)) (Niki, 1987).

lipid radical ($L^{\bullet}$) is formed from the lipid, usually by an attack of radicals, light, heat, irradiation or metal. Initiation has little effect on the subsequent chemistry in the chain reaction, though the rate of initiation controls the extent of the chain reaction. The propagation sequence is started with a rapid reaction between the carbon-centred, lipid radical and oxygen that gives lipid peroxyl radicals. The lipid peroxyl radicals attack other lipid molecules through abstraction of hydrogen to give lipid hydroperoxides and a new lipid radical that starts the propagation sequence over again. Many molecules of lipids may be oxidised to lipid hydroperoxides for every initiation event. Ultimately, the oxidative reactions will stop by various termination reactions, i.e. by bimolecular reactions of two lipid alkyl radicals and two lipid peroxyl radicals to form nonradical products such as ketones and secondary alcohols (Sergent *et al.*, 1999). If the system contains antioxidants, termination can occur by reaction of the alkyl or peroxyl radicals with physiological antioxidants such as α-tocopherol and glutathione. In this case the initial rate of oxidation, called the lagphase, is slow because these antioxidants first scavenge peroxyl radicals, and when the antioxidants are consumed the rate of propagation increases (Sergent *et al.*, 1999). Breakdown of alkoxyl and peroxyl radicals can lead to the formation of many compounds, e.g. hydrocarbons, malondialdehyde, 2-alkenal derivatives, low molecular weight alcohols, esters and hydrocarbons (Sergent *et al.*, 1999).

Importance of transition metals in lipid peroxidation

Transitional metals (Fe, Cu, V and Cd ions) are strongly implicated in the generation of free radicals by decomposition of hydrogen peroxide or lipid hydroperoxide (LOOH) to give hydroxyl radicals or alkoxyl radicals, respectively (Sugihara *et al.*, 1999). This metal-induced decomposition of LOOH is believed to be involved in physiologically important processes. The exact role played by iron ions in accelerating lipid peroxidation is an area of great confusion (Halliwell & Gutteridge, 1989). Although metal ions can induce lipid peroxidation in low-density lipoprotein (LDL), their involvement *in vivo* is still controversial as iron must be "free" i.e., not stored in proteins like hemoglobin, myoglobin, ferritin, hemosiderin or transferrin to be catalytically active (Sergent *et al.*, 1999). These amino acids and proteins scavenge divalent ions in plasma yet advanced arteriosclerotic lesions contain significant amounts of catalytically active copper and iron that can catalyse the oxidation of LDL by macrophages. Iron(II) ions are themselves free radicals and can take part in electron transfer reactions with molecular oxygen. Superoxide can dismute to form hydrogen

peroxide giving all the essential ingredients for Fenton chemistry and the formation of OH radicals (Sergent *et al.*, 1999).

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad \text{(Reduction of metal)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \quad \text{(Fenton reaction)}$$

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet} \quad \text{(Haber-Weiss reaction)}$$

Antioxidant actions against lipid peroxidation

The autoxidation of lipids is usually, though not always, a deleterious process. Although the enzymatic oxidations of lipids are controlled processes, the free radical-mediated chain oxidations of lipids are random events (Niki, 1987). Figure 1 suggested that the autoxidation of lipids can be suppressed either by inhibiting the initiation step or by accelerating the termination step. Antioxidants that are able to prevent lipid peroxidation through these two processes are known as preventative antioxidants and chain-breaking antioxidants, respectively, and may act in several ways by : (Rice-Evans, 1995; Terao & Piskula, 1998)

(i) Preventing chain initiation by scavenging initiating radicals such as $OH^{\bullet}$;

(ii) Chelating metal ions thus depressing the superoxide driven Fenton reaction which is currently considered as the most important route to active oxygen radicals (Afanasév, 1989);

(iii) Scavenging lipid alkoxyl and peroxyl radicals by acting as chain-breaking antioxidants, e.g. as hydrogen donors; and

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$$

$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet}$$

(iv) Regenerating α-tocopherol through reduction of the α-tocopherol radical as a preventative antioxidant.

Antioxidants can decrease the number of oxidised LDL particles formed *in vitro*. Epidemiological studies (Gey, K.F., 1990; Hertog *et al.*, 1993; Hertog *et al.*, 1995; Hollman *et al.*, 1996; Hertog, 1996; Dubick & Omaye, 2001) have shown that phenolic antioxidants, including quercetin in food products, correlate with the decreased incidence of coronary heart disease. High serum concentrations of antioxidant nutrients such as vitamin E have been shown to inhibit the initial oxidation step in lipid peroxidation (Diplock, 1998; German, 1998). The unique action of flavonoid antioxidants in phospholipid bilayers is unlike α-tocopherol or ascorbic acid. Their radical-scavenging activity is much lower than that of α-

tocopherol, a predominant chain-breaking antioxidant in biomembranes. However, the hydrophilic properties of flavonoids facilitate their localisation at the interface of the bilayers and thereby effective inhibition of initial attack by aqueous radicals is expected (Terao & Piskula, 1998).

The protective effect of glutathione (GSH) against Fe^{2+}/ascorbate-induced lipid peroxidation in liver microsomes depends on the presence of α-tocopherol (vitamin E) (Van Acker *et al.*, 2000). Reduced GSH has been shown to protect against peroxidation of lipids in rat liver. GSH seems to be able to regenerate α-tocopherol from the tocopheroxyl radical via a so-called free radical reductase, mainly present in the liver. This recycling process keeps the levels of α-tocopherol high enough to protect the membranes against lipid peroxidation. In agreement with earlier findings Van Acker *et al.* (2000) showed that in α-tocopherol-deficient microsomes GSH did not protect against lipid peroxidation. When α-tocopherol–deficient microsomes were preincubated with flavonoids using concentrations of approximately three times their IC_{50} values of lipid peroxidation inhibition, 7-mono hydroxyethyl rutinioside (monoHER), fisetin and naringenin mimicked the effect of vitamin E reintroduction in which the protective effect of GSH was restored. The concentration of fisetin in the membrane was in the same order of magnitude as α-tocopherol suggesting that fisetin could take over the antioxidant role of vitamin E with a similar potency. Naringenin is much less efficient and very high plasma concentrations would be needed to give an effect. Luteolin was shown to be more effective than quercetin, fisetin, myricetin, eriodictyol, epicatechin and rutin against lipid peroxidation induced by Fenton's reaction (Shimoi *et al.*, 1996).

Arteriogenesis

Biomembranes are composed of phospholipid bilayers with proteins and are one of the major targets of reactive oxygen species. Oxidation caused by reactive oxygen species affects membrane functions by inducing continuous lipid peroxidation (Terao & Piskula, 1998). This uncontrolled reaction in cellular and subcellular membranes causes or amplifies pathological phenomena in degenerative diseases such as cancer or arteriosclerosis (Terao & Piskula, 1998). Arteriosclerosis is not a trivial or rare disease and for people enjoying a Western lifestyle it is the major cause of mortality (Esterbauer *et al.*, 1992).

Arteriosclerosis is a chronic inflammatory process which leads to reductions in the luminal diameter of the main arteries by arteriosclerotic lesions (Luc & Fruchart, 1991).

Early arteriosclerotic lesions are characterised by the presence of fatty streaks, which are composed of so-called foam cells (Esterbauer *et al.*, 1992). Foam cells are occlusions and plaques, formed in the intima regions of the major arteries, that have become altered in appearance by internalised lipids (Esterbauer *et al.*, 1992). The complexity of these arteriosclerotic lesions increases as a function of their age and they are characterised histologically by cellular proliferation, primarily of macrophages and smooth muscle cells with the accumulation of lipids, mainly cholesterol both intra-and extracellularly (Luc & Fruchart, 1991). Foam cells are derived from smooth muscle cells and monocyte-macrophages. Blood monocytes adhere to the arterial endothelium and penetrate into the intima to become resident macrophages in this subendothelial space. They take up lipids and lipoproteins, infiltrated and deposited in those regions.

There is no doubt that elevated plasma cholesterol levels play a dominant role in cardiovascular disease and no-one has been able to generate arteriosclerotic lesions in any animal model without somehow raising plasma cholesterol levels (Witztum & Steinberg, 2001). However monocytes/macrophages firstly have very few LDL receptors and secondly these are down regulated by the accumulation of cholesterol therefore uptake of native LDL (the major cholesterol transport lipoprotein) by way of the Brown/Goldstein LDL receptors does not result in foam cells (Luc & Fruchart, 1991). Although, an increase in plasma LDL levels does lead to an increase in the adherence of circulating monocytes to arterial endothelial cells and at the same time an increased rate of entry of LDL into the intima, resulting in higher steady state concentration of LDL in the intima (Steinberg, 1997). There the LDL can undergo oxidative modification catalysed by any of the major cell types found in arterial lesions, *i.e.* endothelial cells, smooth muscle cells, or macrophages (Steinberg, 1997). Macrophages do have specific cellular receptors for this modified form of LDL called "scavenger receptors". These differ from the classical Brown/Goldstein receptors in that they do not bind native LDL and are not down regulated (Luc & Fruchart, 1991). Thus uptake of oxidised LDL by macrophages takes place uninhibited. Compositional properties of LDL relevant for its susceptibility to oxidation, kinetics of oxidation and consequences for the formation of arteriosclerotic plaques have been extensively studied and reviewed (Witztum & Steinberg, 1991; Esterbauer *et al.*, 1992; Croft *et al.*, 1995; Berliner & Heinecke, 1996; Esterbauer & Ramos, 1996; Kontush *et al.*, 1996).

The "oxidation hypothesis" states that the oxidative modification of LDL, or other lipoproteins, is central, if not obligatory to the arteriogenic process (Witztum & Steinberg, 2001). The important corollary is that inhibition of such oxidation should reduce the

progression of arteriosclerosis, independent of the reduction of the other risk factors, such as elevated LDL levels. It has become abundantly clear that oxidised LDL, with its many oxidatively modified lipids and degradation products, contributes to the pathophysiology of both the initiation and progression of the arteriosclerotic lesion by many mechanisms, including its pro-inflammatory, immunogenic and cytotoxic properties as reveiwed in Navab *et al.* (1996), Steinberg & Witztum (1999) and Tsimikas & Witztum (2000).

For many, the litmus test of the oxidation hypothesis is whether or not administration of antioxidants significantly slows the formation of arteriosclerotic lesions. The hypothesis has passed this litmus test in an impressive array of experimental animal models. In a recent review, 16 out of 23 such studies, revealed inhibitory effects ranging from 30-80%, 2 borderline and 5 negative (Steinberg & Lewis, 1997). These studies have included a number of different animal models, and they have utilised several different antioxidants (Chisolm & Steinberg, 2000). It is significant that striking inhibition has been observed even in the case of extremely high plasma cholesterol levels (>800 mg dL^{-1}), especially in the knockout mouse studies and in the studies in LDL-receptor deficient rabbits (Chisolm & Steinberg, 2000).

Various clinical trials in humans utilising antioxidants such as β-carotene and vitamin E have recorded cardiovascular effects (Steinberg, 1997). In a carefully controlled trial β-carotene however, was shown to have no significant effect in protecting circulating LDL from oxidation (Reaven *et al.*, 1994). Vitamin E, on the other hand, is very effective in protecting circulating LDL against oxidation *ex vivo*. A clinical trial utilising 400-800 IU of vitamin E daily in a placebo-controlled, double blind trial in patients with established coronary heart disease found 47% fewer nonfatal myocardial infarctions and cardiovascular deaths than the control groups, and the result was significant at the $p < 0.0001$ (Stephens *et al.*, 1996). At this time there is insufficient evidence, however, to allow a confident prediction of the anti-arteriosclerotic effectiveness of a compound from its antioxidant effectiveness *ex vivo* (Steinberg, 1997). It appears that some rather high threshold of antioxidant effect must be reached before any anti-atherosclerotic effect is evident.

Ageing

The free-radical theory of ageing was introduced in 1956 by Denham Harman in the United States of America (Halliwell & Gutteridge, 1989). He proposed that normal ageing results from random deleterious damage of tissues by free radicals produced during normal aerobic

metabolism. In support of this concept we know that radiation produces damaging free radicals and accelerates some features of the ageing process, although radiation-induced "ageing" does not exactly mimic normal ageing (Halliwell & Gutteridge, 1989). Oxygen free radicals are produced during normal metabolism but escape scavenging, especially if there is an imbalance, to cause damage. Bruce N. Ames has calculated that in excess of 1000 oxidative 'hits' per cell per day take place upon cellular DNA (Halliwell & Gutteridge, 1989). Much experimental data exists indicating a causative role of oxyradicals in ageing processes. In testing the hypothesis that antioxidants may represent longevity determinant genes, a positive correlation in the tissue concentration of specific antioxidants with life span of mammals has been found (Cutler, 1991). These antioxidants include superoxide dismutase, carotenoids, α-tocopherols, and uric acid. It has also been found that the resistance of tissues to spontaneous autoxidation and the amount of oxidative damage to DNA correlates inversely with life span of mammals. These results suggest a role of oxyradicals in causing ageing and that the antioxidant status of an individual could be important in determining frequency of age-dependent diseases and duration of general health maintenance.

Today it is generally accepted that ageing is related to an increase in oxidation products derived from nucleic acids, sugars sterols and lipids (Spiteller, 2001). The effects of lipid peroxidation are proliferation, wounding and ageing and result in a change in cell wall structure that activates membrane bound phospholipases (Spiteller, 2002). These phospholipases cleave phospholipids resulting in the release of polyunsaturated fatty acids (PUFAs) that are able to serve as substrates for lipoxygenases which leads to the generation of lipid hydroperoxides (LOOHs). However, if the amount of free PUFAs exceeds a certain amount, lipoxygenases commit suicide resulting in the liberation of free iron ions that react with LOOHs resulting in the formation of radicals that start a chain reaction (Spiteller, 2001; Spiteller, 2002). The LOO radicals produced in the course of this process attack proteins, nucleic acids, and also double bonds of all unsaturated compounds by epoxidation. Moreover LOOHs are decomposed to toxic epoxy acids and alpha-, beta-, gamma-, delta-unsaturated aldehydes (Spiteller, 2002). Both species react with glutathione and the resulting products seem to induce apoptosis (Spiteller, 2002).

Mutagenesis and cancer

Cancer is a serious health problem in many countries and is accountable for over 7 million deaths per year world wide (Tanaka, 1997). In Western countries, the incidence of cancer is second only to cardiovascular disease as the most frequent cause of death (Chen *et al.*, 1983).

The first description of occupational cancer was in 1775. It was Percival Pott who, being a proficient surgeon, had noticed that many chimneysweepers had cancer of the scrotum and testicles. He supposed that this disease originated from the lodgement of soot in the rugae of the scrotum (Pott, 1775). In fact, through Pott's observation, the way was opened to the prevention of this disease and to the isolation and synthesis of the first known pure carcinogen, benz[*a*]pyrene (B[*a*]P). It took over a hundred years, however, before results of experimental research were published showing the causal relation between exposure to chemicals and cancer. Its discovery and the extraordinary proliferation of man-made chemicals in the environment have motivated extensive research into this field by regulatory authorities, academics and industrialists.

Cancer is believed to occur via a multi-step mechanism that consists of an initiation, promotion and progression phase (Figure 2). The first step in carcinogenesis, initiation, is characterised by mutations in the DNA of chromosomes (Newell, 1981; Kuroda & Hara, 1999). This mutation cannot be the sole mechanism for cancer due to the very rapid process of mutational change that can be followed by a long latent period, of 30 years or more before induction. Therefore the mutational theory of carcinogenesis is only applied to the initiation phase and is followed by a promotional and progression phase related to phenotypic expression (Sugimura *et al.*, 1981; Kuroda & Hara, 1999). The promotional phase that follows initiation, results in abnormal proliferation that occurs due to altered enzyme activity and metabolism in the cell membrane and the cytoplasm (Kuroda & Hara, 1999). This is then followed by the final step in carcinogenesis called progression, where malignant cells grow and invade into the surrounding normal tissue or organs (Kuroda & Hara, 1999).

Environmental mutagens and their modes of action

The process of mutagenesis can be brought about by two types of mutagens namely, direct-acting mutagens and promutagens. Direct-acting mutagens, which are found mostly in a reactive form, do not require metabolic activation in order to cause initiation or mutational change in the genome. Promutagens or procarcinogens require metabolic activation before they are able to induce mutagenesis (Sugimura *et al.*, 1981). Once activated, procarcinogens

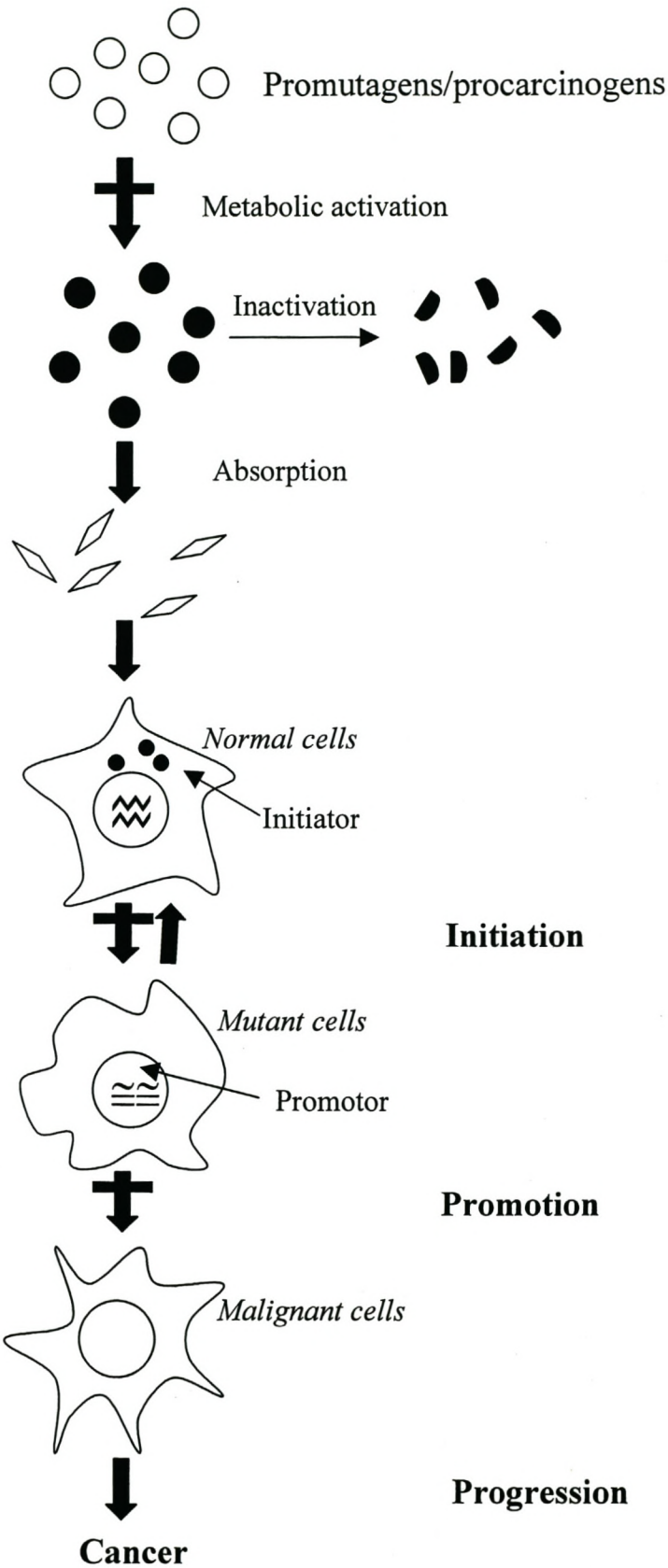


Figure 2. Multistep carcinogenesis (Kuroda & Hara, 1999).

become electron-poor (electrophilic reactant) and can then bind with electron-rich areas of the cells, such as the nucleic acids (DNA and RNA) and cellular proteins (Newell, 1981).

A number of mutagenic compounds exist in the diet, some of which are formed during cooking. Mutagens can be formed in muscle foods when subjected to various cooking and processing methods. Benz[*a*]pyrene, a representative carcinogenic polycyclic aromatic hydrocarbon and heterocyclic amines exist in exhausts, tar of tobacco, charred fish and meat, and are well known mutagens (Nakasagi *et al.*, 2000). These mutagens undergo metabolic activation by P_{450} enzymes, especially the CYP1A enzyme family, involving hydroxylation and epoxide formation at the 7,8 and 9,10 positions and the resultant carcinogenic metabolites form covalent bonds with DNA causing mutations (Namiki, 1990; Nakasagi *et al.*, 2000). Ikeda *et al.* (1983) reported that people who frequently eat charred fish that contain the moderate heat temperature-induced 2-aminoimidazole-type mutagens such as the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Chen *et al.*, 1983) have a higher incidence of gastric cancer.

The mutagens that have been isolated from cooked foods can be classed into two groups of heterocylic amines, the 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) type and the non-IQ type. 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) belong to these groups respectively (Yamagishi *et al.*, 2000). IQ, MeIQ and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) are potent frameshift mutagens and have been found to be much more potent than 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), Trp-P-2, 2-amino-6-methyldipyrido[1,2-α:3',2'-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-α:3',2'-*d*]imidazole (Glu-P-2). MeIQ has been implecated as one of the most potent mutagens that has been tested in the Ames *Salmonella* bacterial mutagenesis assay (Chen *et al.*, 1983).

Ingestion of foods contaminated with the mycotoxin aflatoxin (AF) is an important health risk factor in developing countries (Key *et al.*, 2002). The rates of liver cancer vary greatly between countries and are much higher in sub-Saharan Africa and southeast Asia than in Europe and North America (Key *et al.*, 2002). Mycotoxins are secondary metabolites of molds that have adverse effects on humans, animals, and crops that result in illnesses and economic losses. Aflatoxins, ochratoxins, trichothecenes, zearelenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance (Hussein & Brasel, 2001). Aflatoxins are the most studied (5000 publications) group of mycotoxins and are produced by different species of the genus *Aspergillus* (Hussein

& Brasel, 2001). Variations in the magnitude of toxicity exist among AF. For example, AFB_1 is the most toxic in both acute and chronic aflatoxicoses whereas AFM_1 (i.e. a metabolite in milk) is as acutely hepatotoxic as AFB_1 but not as carcinogenic (Carnaghan *et al.*, 1963). The activated AFB_1 metabolite (i.e. AFB_1-8,9-epoxide) result in GT transversions by forming a covalent bond with the N7 of the nucleotide guanine (Lillehoj, 1991).

Aromatic amines are carcinogenic in experimental animals and epidemiological studies have shown them to be carcinogenic in humans (Shibutani & Grollman, 1997). 2-Acetylaminofluorene (2-AAF), an aromatic amide, is widely used as a model chemical carcinogen (Hadjiolov *et al.*, 1995). Chronic feeding of the carcinogenic arylamine 2-AAF produces liver tumours in rats. 2-AAF is a complete liver carcinogen in these species therefore tumours appear. Following metabolic activation, 2-AAF reacts with cellular DNA to form covalent adducts, including N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF), N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF), and N-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AAF). dG-C8-2AAF is the major adduct found when DNA is treated with 2-AAF in vitro, however, dG-C8-AF is the predominant adduct recovered from 2-AAF treated cells. Five to fifteen percent of DNA bound aminofluorene is represented by dG-N^2-AAF that is a persisting lesion in animal tissues while dG-C8-AAF and dG-C8-AF are repaired more rapidly (Hadjiolov et al., 1995).

Antimutagenesis and antimutagens

Since mutagenesis is defined as a heritable change that can occur through indirect as well as direct change in the genetic message, antimutagenesis can be defined as the decrease in mutation frequency regardless of the mechanism involved (Singer, 1982; Kada & Shimoi, 1987). Antimutagens can be classified into various categories according to their modes of action. Kada & Shimoi (1987) proposed a distinction among categories of antimutagens. Antimutagens that inactivate mutagens before they attack DNA *in vitro* were defined as desmutagens and those that suppress the fixation process required before damaged genes can be expressed as stable and heritable genes as bio-antimutagens (Figure 3).

Diet and cancer

It was soon discovered that only a small proportion of cancers are actually inherited through the gene line as single-gene defects or chromosomal aberrations that predisposed an individual to develop certain forms of cancer. Although more than 200 single-gene disorders

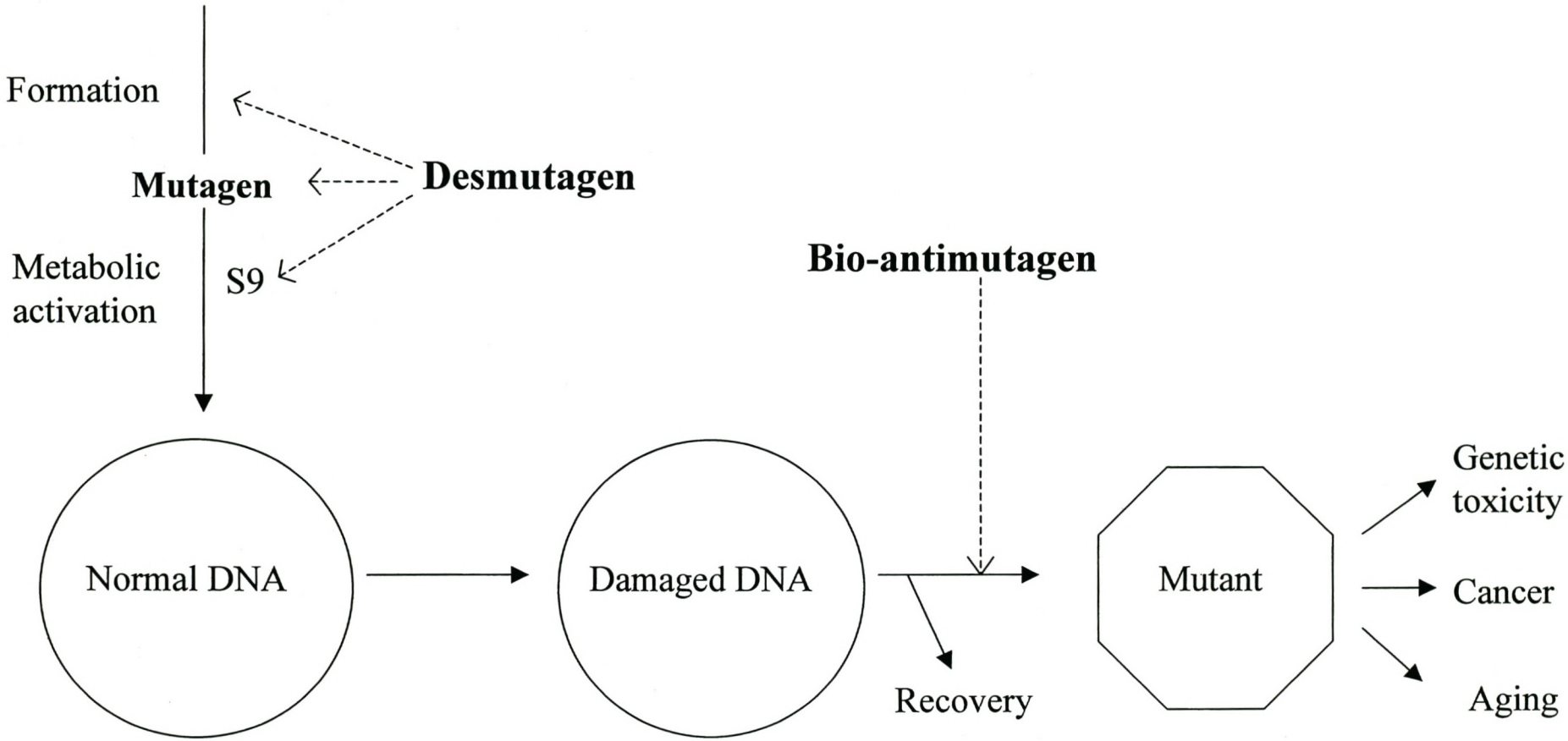


Figure 3. Schematic action of desmutagens and bio-antimutagens (Kada & Shimoi, 1987)

have been associated with cancers, they account for less than 5% of all malignancies (Newell, 1981). In general, cancers associated with hereditary syndromes occur less frequently than do sporadic nonhereditary occurrences of the same cancers (Newell, 1981). As many as 70-90% of human cancers have been estimated to be associated with environmental causes (Doll & Peto, 1981).

Environmental causes of cancer are often misunderstood and misconstrued as being primarily due to ubiquitous chemicals derived from modern technology and industrial development. It is true that a number of food additives, pesticides, insecticides and industrial chemicals introduced commercially in the past 40 years have exhibited carcinogenic properties in animal models (Chen *et al.*, 1983). However, the main causes of human cancer in the Western world do not stem from such chemical contaminants. Epidemiological studies have shown that diet and lifestyle are closely related to human cancer (Chen *et al.*, 1983). For instance Haenszel *et al.* (1973) illustrated that cancer of the stomach is more common in Japan, whereas in the United States cancer of the large intestine, the breast and the prostate are more common. It was shown that when Japanese immigrate to the United States, these differences were lost within a generation or two. The causative agent for this was diet which tends to persist as part of a cultural heritage and not factors such as air pollution that tend to be the same for everyone in a given place. The cancer could further be related to diet and not to genetic make-up due to the tendency of immigrants to marry within groups and the incidence of cancer taking more than a generation genetically to reach levels typical of the United States (Haenszel *et al.*, 1973).

With people becoming more aware of the prevalence of diet related cancers there has been a movement towards healthier eating and away from the use of synthetic chemicals. The rising interest in functional foods has prompted research into foods with natural health benefits one of which being the antimutagenic actions of flavonoids and phenolic compounds (Hardigree & Epler, 1978; Kada *et al.,* 1985; Wang *et al.,* 1989; Yen & Chen, 1995; Yen & Chen, 1996; Yen & Chen, 1997; Kuroda & Hara, 1999; Yamagishi *et al.*, 2000).

Antimutagenic actions of flavonoids

Plants contain a wide variety of compounds that have antimutagenic properties and appear to act as antioxidants. Flavonoids, have been studied extensively for their anticarcinogenic properties (Choi *et al.*, 1994; Nakasugi & Komai, 1998; Nakasugi *et al.*, 2000). Since the generation of reactive oxygen species (ROS) appears to correlate with tumour promotion, reduction of the radicals and oxidants with antioxidants (flavonoids) would be expected to

decrease tumour promotion activity (Pryor, 1994). Animal experiments have generally supported this concept in that antioxidants are most protective during the promotional phases of cancer development (Pryor, 1994).

The presence of antimutagenic compounds in teas has been reported in various studies under different conditions (Wang *et al.*, 1989; Apostolides *et al.*, 1996; Chen & Yen, 1997). Apostolides *et al.* (1996) reported that black tea polyphenols are more potent inhibitors of the mutagenicity of PhIP in TA98 with S9 activation in the *Salmonella typhimurium* assay than the polyphenols of green tea. Chen & Yen (1997) observed no effect when testing green and black tea for bio-antimutagenic effects against the mutagens PhIP and B[*a*]P to TA98. They found all the tea extracts (oolong, green and black) showed desmutagenic effects with the amount of inhibition of mutagenicity increasing with an increased pre-incubation period (mutagen, S9 and tea extract).

Regarding the specific mechanism involved in antimutagenic action of teas Wang *et al.* (1989) indicated that enzyme activity in cytochrome P_{450} would be inhibited by binding with catechin in green tea. The inhibition of P_{450} enzyme activity might lead to inhibition of metabolic activation of the mutagen concerned and their subsequent binding to DNA and/or their mutagenic potency. Tea extracts may exert their antimutagenic action by more than one mechanism i.e. interaction of tea extracts with promutagens, S9 mix and the activated metabolites of these mutagens. They may enhance repair of DNA damage and/or interact with mutagen metabolites (Chen & Yen, 1997). For this reason the antimutagenicity assay used should be able to measure not only the ability of a compound to act as a antimutagen but also to distinguish whether the compound can be classed as a desmutagen or bio-antimutagen against promutagens or direct acting mutagens (Chen & Yen, 1997).

Bioavailability and absorption of flavonoids

Bioavailability is of major interest in assessing the biological effects of flavonoids or indeed of any food component or drug through quantitation of the exposure of the body to the substance in question. It can be defined as the percentage of the ingested flavonoid amount that enters the blood circulation intact after passage through the liver (Hollman & Katan, 1998). Since bioavailability is often mistakenly equated with absorption it is important to note that it also includes first-pass metabolism (Hollman & Katan, 1998).

Critical to any discussion regarding the efficacy of tea polyphenols in the prevention of arteriosclerosis and heart disease, is how much is consumed in a typical diet, how efficiently such compounds are absorbed through the gastrointestinal tract, as well as

information regarding their tissue distribution, metabolism and rate of elimination (Dubick & Omaye, 2001). Unfortunately there is limited information on humans, which has led to uncertainty that these compounds could express *in vivo* antioxidant activity of physiologic significance (Dubick & Omaye, 2001). Because such compounds occur as complex mixtures in plant materials and due to their enormous variability, it is difficult to study bioavailability and physiologic effects. Dubick & Omaye (2001) have summarised the factors that may contribute to the difficulty in assessing the metabolism and bioavailability of polyphenols in the diet as:

(i) Uneven distribution of polyphenols in food;

(ii) Species related polyphenol content;

(iii) Effect of food processing;

(iv) The role of gut microflora; and

(v) Interactions with other food components (including alcohol).

Early studies on flavonoid metabolism have concentrated on the acid fission products resulting after microbial cleavage at the heterocyclic ring by intestinal bacteria and it was generally thought that the absorption of a flavonoid glycoside could not take place until the compound reached the microflora of the large intestine where cleavage of the β-glycosidic bond could occur (Hacket, 1986). However, recent studies have demonstrated the presence of quercetin-3-rhamnoglucoside (rutin) in the circulation after the consumption of apple or onion and in the blood plasma of individuals consuming a generally high fruit and vegetable diet (Hollman *et al.*, 1997; Paganga & Rice-Evans, 1997). This was also demonstrated in ileostomy subjects where the absorption of quercetin-β-glucosides (such as quercetin-4'-glucoside) from onions was 52%, compared to that of quercetin without its sugar moiety, the so-called aglycone, and quercetin-β-rutinoside that were both only about 20% (Hollman *et al.*, 1995). This contradicts the theory that flavonols from the diet are first hydrolysed by the digestive microflora before being absorbed since here the glycosides were more readily absorbed than the aglycone form. From the study by Hollman *et al.* (1995) it was concluded that conjugation with glucose enhances absorption from the small gut and that, with repeated consumption of quercetin-containing foods, quercetin will accumulate in the blood since the half-lives of elimination were determined as 28 h for onions and 23 h for apples. With the removal of the rhamnose molecule, with the enzyme alpha-*L*-rhamnosidase, from the rutinose dimer of quercetin-3-rutinoside (the major form of quercetin in black tea) the resulting bioavailability of the product, quercetin-3-glucoside, equals that of quercetin-4'-glucoside

(the major form of quercetin in onions) (Olthof *et al.*, 2000). Therefore the position of glucose conjugation does not interfere with the bioavailability and through removal of the rhamnose moiety from flavanol rutinosides the bioavailability may be enhanced (Olthof *et al.*, 2000).

In addition to the increased absorption of flavonoids conjugated with glucose there is growing evidence that glucoronidation is central to flavonoid metabolism especially for hydroxylated flavonoids (Oliveira & Watson, 2000). In order for glycosides such as luteolin-7-glucoside, kaempferol-3-glucoside and quercetin-3-glucoside to appear as glucuronides of their aglycones, the glycosidic groups must be cleaved by an intestinal enzyme prior to glucuronidation. Apart from the glucuronidation that occurs in the small intestines and liver (Rice-Evans & Rechner, 2000) it has been shown that these compounds are also cleaved and glucuronidated by rat jejunal or ileal mucosa, suggesting the presence of a glucosidase, and a UDP glucuronyl transferase that glucuronidates the phenolics before efflux into the serosal fluid (Spencer *et al.*, 1999). The findings of this study with regard to the perfusion of luteolin and quercetin glycosides and aglycones as glucuronides (90-100% of the total present in the serosal fluid) through sections of rat jejunum were in agreement with those of Shimoi *et al.* (1998). Glucuronidation of the flavonoids was observed to occur at different and possibly multiple hydroxyl groups within the structure (Spencer *et al.*, 1999). The position at which the glucuronidation occurs will be of importance when considering the resulting antioxidant potential of the absorbed glucuronides especially since the reduction potential of the B-ring of phenolics is lower than that of the A-ring (Spencer *et al.*, 1999). For instance the antioxidant capacity of 3'-*O*-methylquercetin and conjugated derivatives of quercetin (Figure 4) have a prolonged lag phase on the inhibition of Cu^{2+}-induced oxidation of human LDL, but the magnitude of their effect is about half that of the aglycone (Manach *et al.*, 1998). Recently Moon *et al.* (2001) demonstrated for the first time that quercetin-3-*O*-β-*D*-glucuronide accumulates in rats *in vivo* after oral administration. The concentration of quercetin-3-*O*-β-*D*-glucuronide in rat plasma 30 minutes after administration of 50 mg aglycone.200 g^{-1} body weight was found at 4.2 μM. The total quercetin metabolites one hour after administration was 9.6 μM. Metabolites have been found to accumulate in human plasma in the concentration range of $10^{-7} - 10^{-6}$ M with continuous ingestion of quercetin-rich foods (Moon *et al.*, 2000). Since the level of quercetin-3-*O*-β-*D*-glucuronide required to inhibit copper-ion induced lipid peroxidation in human LDL was within this concentration

range it is conceivable that adequate protection against LDL oxidation can be provided through a quercetin rich diet.

Isoflavone absorption and metabolism is thought to greatly depend on the bacterial composition in the large intestine (Wiseman, 1999). The action of the bacteria can result in oestrogenic metabolites, in the case of daidzein metabolised to equol or non-oestrogenic metabolites, such as the metabolite of genistein, *O*-demethylangolensin (Wiseman, 1999). The paramount role of the intestinal bacteria in the metabolism of lignans and isoflavones was discovered by the observation that germ-free animals fed diets rich in these compounds did not excrete any of the isoflavone metabolites equol, enterodiol or enterolactone in urine (Axelson & Setchell, 1981). These compounds were not detectable in blood or bile. In addition, antibiotic administration to humans also abolished their formation (Setchell *et al.*, 1981). In human studies conducted to date, only a maximum of approximately 20-30% of the ingested isoflavone has been accounted for by urinary excretion of aglycones and known metabolites. Plasma concentrations in the range of 50 to 800 $ng.mL^{-1}$ have been reported in adults after consumption of 50 mg isoflavones per day (Cassidy *et al.*, 2000).

With regard to tea polyphenols and proteins, especially those found in milk conflicting evidence has been found on the absorption of flavonoids after complexation with the milk proteins. Serafini *et al.* (1996) and Hollman & Katan (1998) found that ingestion of tea by humans caused a significant increase of the plasma antioxidant capacity, but not when tea was consumed with milk. These authors however, did not quantify the polyphenols in the plasma they merely determined the antioxidant capacity of the plasma. Van het Hof *et al.* (1998) and Hollman *et al.* (2001) later opposed this with the discovery that the addition of milk did not in fact affect the bioavailability of the flavonols in humans after quantification of the plasma concentrations of flavonols. It was Arts *et al.* (2002) who then set out to explain the interactions between flavonoids and proteins and the effect on total antioxidant capacity. In this study it became evident that the antioxidant capacity of several components of green and black tea complexed with α-, β-, and κ-casein or albumin was not additive, since they posses certain degrees of individual activity, indicating that a part of the antioxidant capacity was masked by the interaction. This masking was shown to depend on both the protein and the flavonoid used. Compounds in green and black teas that were shown to have the highest masking in combination with β-casein were (-)-epigallocatechin 3-*O*-gallate and gallic acid.

Structure-activity relationships of flavonoids and isoflavonoids with regard to antioxidant and antimutagenic activity

Flavonoids have a diphenylpropane ($C_6C_3C_6$) skeleton and individual differences within each group result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation of these groups. Although there is a wealth of data on the importance of antioxidants and antimutagens in conferring stability towards or protection from oxidation, the correlation between antioxidant activity and chemical structure is far from clear. Different methods of assessment, varying substrate systems, and differential concentrations of active antioxidants and antimutagens all have contributed to confounding the issue. This section will review current views on the structure-activity relationships of antioxidative and antimutagenic flavonoids.

Antioxidant structure-activity relationships

In the aqueous phase the effective radical scavenging by compounds can be summarised into three criteria (Bors *et al.*, 1990; Sichel *et al.*, 1991):

(i) The ortho-dihydroxy structure in the B ring, which confers higher stability to the radical form and participates in electron de-localisation;

(ii) The 2,3-double bond in conjugation with a 4-oxo function in the C ring, responsible for electron delocalization from the B ring (phenoxyl radicals produced are stabilised by the resonance effect of the aromatic nucleus); and

(iii) The 3- and 5-OH groups with the 4-oxo functions in A and C rings, for maximum scavenging potential.

Quercetin (Figure 4) satisfies all of the above-mentioned criteria and is the most effective of the different flavonoids except epigallocatechin gallate (Rice-Evans & Miller, 1998). When the two hydroxyl groups appear in a *meta*-position to each other on the B ring the antioxidant activity is drastically reduced (2.55 mM for morin as compared to 4.7 mM for quercetin with an *ortho*-diphenolic arrangement) (Table 1). The addition of a third OH group in the B ring, as for myricetin or the presence of a single OH group, as for kaempferol also leads to a decrease in the antioxidant activity (3.1 mM and 1.34 mM respectively) when compared with quercetin. The antioxidant activity of the flavones is less than their corresponding flavonols due to the absence of the 3-OH (2.1 mM for luteolin compared to 4.7 mM of quercetin). The effect of the saturation of the 2,3-double bond is illustrated for the flavonol quercetin compared to the flavanone taxifolin.

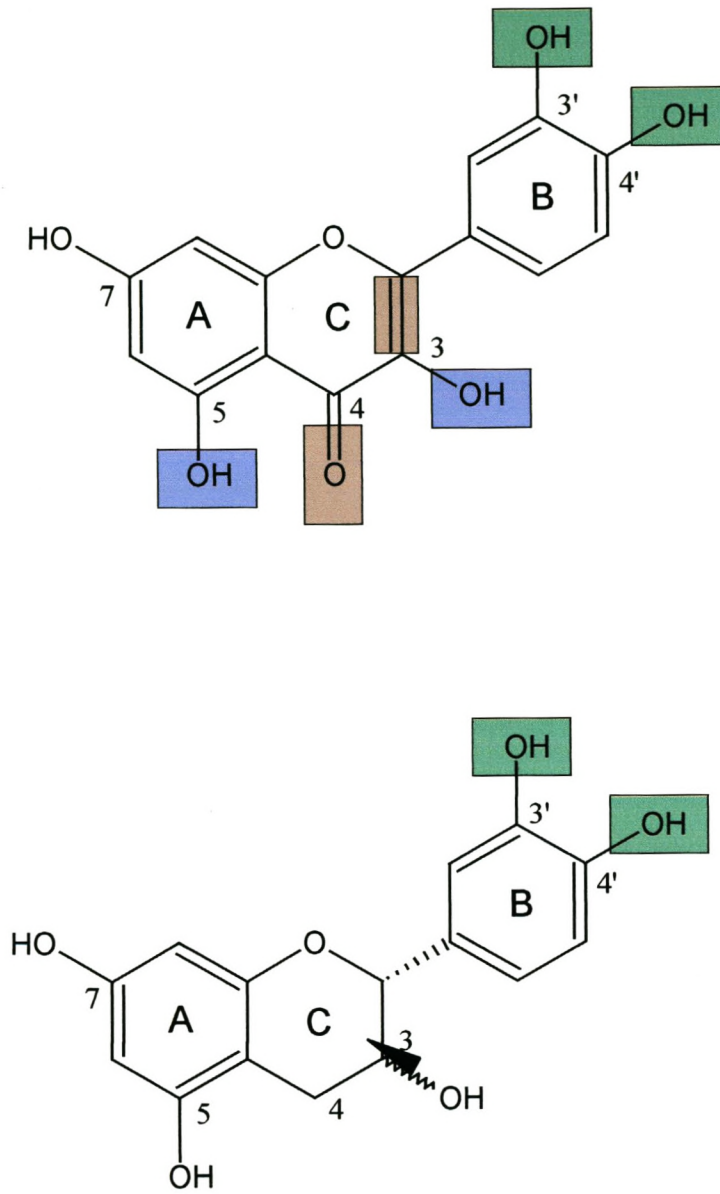
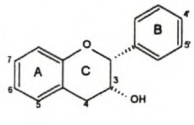
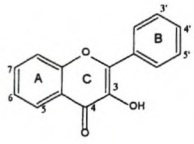
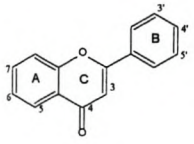
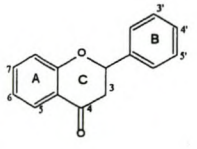
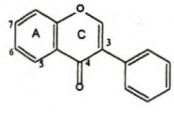


Figure 4. Illustration of structure-activity relationship of quercetin (top) and catechin (bottom) (Rice-Evans & Miller, 1998).

Table 1. Hierarchy of Trolox equivalent antioxidant activities (TEAC) (Rice-Evans *et al.*, 1996) and ability to inhibit rat liver microsomal lipid peroxidation (%I) (Mora *et al.*, 1990).

Family	Compound	Free OH-substitutes	Glycosylated position	TEAC (mM)	% I (100μM)
Flavanol	Epicatechin gallate	5,7,3',4',3",4",5"		4.9 ±0.02	
	Epigallocatechin gallate	5,7,3',4',5',3",4",5"		4.8 ± 0.06	
	Epigallocatechin	5,7,3',4',5'		3.8 ± 0.06	
Flavonol	Quercetin	5,7,3',4'		4.7 ± 0.1	98
	Myricetin	5,7,3',4',5'		3.1 ± 0.3	
	Morin	5,7,3',5'		2.55 ± 0.02	98.2
	Rutin	5,7,3',4'	3-rut	2.4 ± 0.06	98.9
	Kaempferol	5,7,4'		1.34 ± 0.08	98.7
Flavone	Flavone				10.8
	Vitexin	5,7,4'	8-gluc		8.1
	Luteolin	5,7,3',4'		2.1 ± 0.05	97.1
	Luteolin-4'-glucoside	5,7,3'	4'-gluc	1.74 ± 0.09	
	Luteolin-3',7-diglucoside	5,4'	3',7-digluc	0.79 ± 0.04	
	Apigenin	5,7,4'		1.45 ± 0.08	74.5
	Chrysin	5,7		1.43 ± 0.07	96.4
Flavanone	Taxifolin	5,7,3',4'		1.9 ± 0.03	
	Eriodictyol	5,7,3',4'			94.2
	Naringenin	5,7,4'		1.53 ± 0.05	4.4
	Hesperetin	5,7,3'	4'-OMe	1.34 ± 0.08	6
	Hesperidin	5,3'	4'-OMe 7-rut	1.08 ± 0.04	
Isoflavone	Formononetin	7	4'-OMe	0.11	
					

Catechin is similar in structure to quercetin but lacks the 4-oxo function and the 2,3-double bond in the C-ring and therefore has no electron delocalisation between the A and B rings due to the saturation of the heterocyclic ring (Figure 4). Catechins with three hydroxyl groups in the B ring are the gallocatechins and those esterified with gallic acid at the 3-OH group in the C-ring are the catechin gallates. The catechin gallates have a higher antioxidant activity than the gallocatechins since the antioxidant activity of the catechins responds broadly to the tenet that the structures with the most hydroxyl groups exert the greatest antioxidant activity (Rice-Evans *et al.*, 1996). The catechin-gallate esters reflect the contribution from gallic acid (3,4,5-trihydroxybenzoic acid) by the increase in antioxidant activity of 3.8 mM of epigallocatechin to 4.8 mM of epigallocatechin gallate.

Glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Shahidi & Wanasundara, 1992). Blocking of the 3-hydroxyl group in the C ring of quercetin with a glycoside, while retaining the 3',4' dihydroxy structure in the B ring as in rutin caused a decrease in activity to 2.4 mM. Glucosylation of luteolin at the 4' position in the B ring also resulted in a decrease in activity from 2.1 mM (luteolin) to 1.74 mM (luteolin-4'-glucoside).

The basic structural difference between the flavones and the isoflavones is the location of the B ring at the 3-position of the C ring in the case of isoflavones, altering the chemistry of the relationship across the rings, but also removing the 3-OH from the structure. This has a gross influence on the antioxidant activity. There is a minimal contribution to antioxidant activity from the single hydroxyl group on the A ring as can be observed by the antioxidant activity of formononetin (0.11 ± 0.02 mM). This illustrates the strong influence of the diphenolic conformation in the A ring on the antioxidant activity of isoflavones (Rice-Evans & Miller, 1998).

In the lipophilic phase the specific mode of inhibition of oxidation by the individual polyphenolic is not clear but they may act by (Mora *et al.*, 1990):

(i) Chelating copper ions via the ortho dihydroxy phenolic structure;

(ii) Scavenge lipid alkoxyl and peroxyl radicals by acting as chain breaking antioxidants; or

(iii) Regenerating α-tocopherol through reduction of the α-tocopheroxyl radical.

The comparison of a range of flavanones and flavones in their capacity to increase the induction period of the autoxidation of fats has led to the conclusion that optimum antioxidant activity is associated with such structural features as, multiple phenolic groups,

especially the 3',4'-*ortho*-dihydroxy configuration in the B ring (Cotelle *et al.*, 1996), the 4-carbonyl group in the C ring, a free 3-OH group or free 3- and 5-OH groups (Hudson & Lewis, 1983). Possible sites for attachment of divalent metal ions are the catechol moiety in the B ring, the 3-OH, 4-oxo groups in the heterocyclic ring and the 4-oxo, 5-OH groups between the heterocyclic ring and the A ring (Figure 5) (Pietta, 2000). The phenoxyl radical formed by reaction of a phenolic antioxidant with a lipid radical is stabilised by delocalization of unpaired electrons around the aromatic ring, while the resulting radical form is stabilised by the *ortho*-dihydroxy groups. In contrast with the aqueous phase interactions the 2,3-double bond has been shown to not be of importance because taxifolin is more effective than its unsaturated analogue quercetin and in contrast to this Mora *et al.* (1990) has shown that hydrogenation of the 2,3 double bond of apigenin and luteolin to naringenin and eriodictyol, respectively decreased the activity. Catechin lacking the 4-carbonyl group as well as the 2,3-double bond is also relatively ineffective.

A study on the affinity of flavonoids for a biological hydrophobic environment was done through quantification of their affinity for lipid vessicles (Van Dijk *et al.*, 2000). The planar configuration of flavonols strongly favours, in contrast to the tilted conformation of the flavanones, the intercalation of the flavonols into the organised structures of the phospholipids within the vessicle membranes. For the interaction with vessicles, a planar configuration of flavonoids appears of far greater importance than the relative hydrophobicity index as determined by water-oil partitioning. Van Dijk *et al.* (2000) showed that the glycosylation of both naringenin and eriodictyol at the 7 position resulted in a more planar configuration and a higher affinity for vesicle membrane compared to their corresponding aglycones.

Antimutagenic structure-activity relationships

A study by Edenharder & Tang (1997) compared the antimutagenic properties of 56 flavonoids, 32 coumarins and other phenolic compounds with respect to the mutagenicity induced by 2-nitrofluorene, 3-nitrofluoranthene and 1-nitropyrene in *Salmonella typhimurium* TA98. Distinctive structure-activity relationships were detected. Ten out of the fifteen glycosides were totally inactive and four flavonoid glycosides that did exert antimutagenicity did so to a lower degree than their corresponding aglycones. Choi *et al.* (1994) also stated that glycosylation would result in a decrease in antimutagenic activity. Glycosylation of quercetin at position 3 to produce isoquercitrin and rutin resulted in a decrease of antimutagenic activity towards AFB_1 from 55% to 13% and 0% respectively (Table 2).

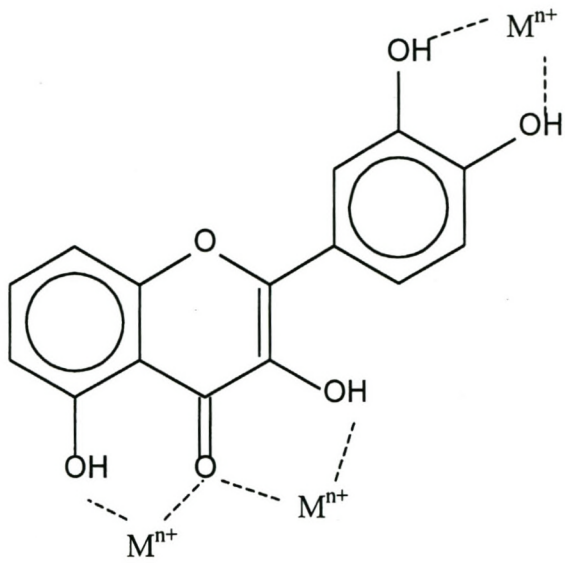
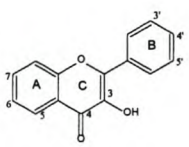
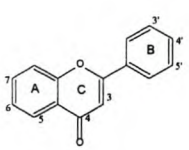
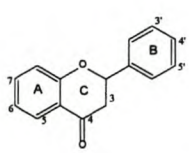


Figure 5. Binding sites for trace metals of flavonoids (Pietta, 2000).

Table 2. Antimutagenic effects of flavonoids and their glycosides against aflotoxin B_1 (AFB_1) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in *Salmonella typhimurium* TA100 and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Tryp-P-2) in *Salmonella typhimurium* TA98.

Family	Compound	Free OH-substitutes	Glycosylated position	% Inhibition		
				AFB_1[a]	MNNG[a]	TRP-P-2
Flavonol	Quercetin	5,7,3',4'		55%	2%	
	Fisetin	7,3',4'		80%	31%	
	Morin	5,7,3',5'		82%	8%	
	Isoquercitrin	5,7,3',4'	3-O-glucose	13%	17%	
	Rutin	5,7,3',4'	3-rutinose	0%	13%	
	Kaempferol	5,7,4'		75%	14%	
	Flavone			51%	79%	
Flavone	Luteolin	5,7,3',4'		89%	7%	94.5%[b]-96.2%[c]
	Cynaroside	5,7,3',4'	7-O-glucose	80%	-14%	
	Diosmin	5,3'	4'-OMe, 7-O-rutinose	3%	31%	
	Apigenin	5,7,4'		88%	6%	86%[b]-93.3%[c]
	Chyrisin	5,7		90%	-12%	
Flavanone	Naringenin	5,7,4'		85%	9%	
	Naringin	5,4'	7-hesperidose	10%	-25%	
	Hesperetin	5,7,3'	4'-OMe	88%	20%	
	Hesperidin	5,3'	4'-OMe 7-rutinose	-21%	24%	
	Hesperetin-5-glu	7,3'	5-glucose	49%	8%	
	Taxifolin	5,7,3',4'		59%	27%	

[a] 300 μg.plate^{-1} of flavonoid solutions was employed for the test (Choi *et al.*, 1994).
[b] 50 μg.plate^{-1} of flavonoid solutions was employed for the test (Nakasugi *et al.*, 2000).
[c] 100 μmol.plate^{-1} of flavonoid solutions was employed for the test (Nakasugi & Komai, 1998).

Positive correlations were found between antimutagenic potency and the polarity of a molecule with the existence of an optimum of activity within flavonols (Edenharder & Tang, 1997). In the flavone, flavonol and flavanones reduction of polarity through methylation always reduced antimutagenicity. The parent compounds flavone and flavanone were inactive, but all flavones with phenolic hydroxyl groups exerted antimutagenicity. Antimutagenic potency against AFB_1 in TA100 reached a maximum with the presence of four hydroxyl functions (luteolin and kaempferol), though the position of hydroxyls was also a determinant of antimutagenic potency. Quercetin and morin differing only by the meta arrangement of the hydroxyl groups in morin compared to the ortho arrangement in quercetin had significantly different activities (Choi *et al.*, 1994). Morin had an activity of 82% compared with that of quercetin (55%) against AFB_1 in TA100. Kaempferol as opposed to morin has a hydroxyl group in the 4' position and not the 3' and 5' positions and has antimutagenic activity against AFB_1 in TA100 of 75%. The keto function at carbon 4 of the flavonoids was essential for antimutagenicity against mutagenesis induced by 2-nitrofluorene, 3-nitrofluoranthene and 1-nitropyrene in tester strain TA98 (Edenharder & Tang, 1997). Flavanols and anthocyanidins, both lacking these keto groups, showed no antimutagenic activity, while many of the flavones, flavanones, flavonol, isoflavonoids and chalcones were potent antimutagens. Antimutagenic potency against aflatoxin B_1, however, was not affected upon saturation of the 2,3-double bond or elimination of the 4-keto group (Choi *et al.*, 1994).

4. POTENTIAL OF HONEYBUSH TEA AS A FUNCTIONAL BEVERAGE

Background

Teas prepared from *Camellia sinensis* have been considered to be "functional foods" in China for centuries (Wiseman *et al.*, 1999). One of the legends surrounding the origins of tea concerns the Chinese emperor Shen-Nung who lived around 1700 BC. While heating some water to allay stomach pains due to overindulgence at dinner, the emperor did not notice that some leaves from a nearby shrub had dropped into the boiling water. He drank the brew and found that it not only tasted delicious but that it also caused the spontaneous disappearance of his stomach pains, thereby demonstrating for the first time beneficial health effects of tea (Wiseman *et al.*, 1999).

Indigenous teas are well known in South Africa, particularly in parts of the southern and south western Cape province where they occur. "Heuningtee, heuningbostee, bergtee and

vleitee" all refer to what South Africans know as honeybush tea but also more specifically to the various species of *Cyclopia.* Originally the name "heuningtee" or "heuningbostee" (honeybush tea) referred to the species *Cyclopia genistoides* and not to the now well known and commercially grown *C. intermedia* (Van Wyk, 1997). *Cyclopia intermedia* or so-called "bergtee" (mountain tee) is a multi-branched woody shrub that grows up to a metre in height. The young twigs have a characteristic golden colour and in the event of fire the plant is able to regenerate by sprouting from its woody base. "Vleitee" or *C. subternata* is also grown commercially but requires more attention since this species regenerates from seed after fire. Both *C. intermedia* and *C. subternata* generally have a more acceptable taste than the less sweet taste of *C. genistoides.* For many years the small-scale production of honeybush tea from plants harvested in the wild, has been sufficient to supply the local demands of the South African market, but growing interest from oversease markets has led to a demand for much larger quantities of a higher quality product (E. Joubert, ARC Infruitec-Nietvoorbij, South Africa, personal communication, 2001). This has resulted in research into cultivation (Coetzee, 2000), optimisation of processing procedures (Du Toit & Joubert, 1998) and the *in vitro* antioxidant (Hubbe, 2000) and antimutagenic properties (Marnewick *et al.*, 2000) of honeybush tea.

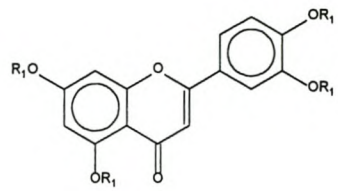
Honeybush tea has been associated with several positive beneficial health effects. Anecdotal evidence suggests that honeybush is a diuretic, can increase appetite, prevent stomach ulcers through stimulation of excess mucous production and stimulate milk production in lactating woman (Watt & Breyer-Brandwijk, 1932). It has also been used to treat colic in babies and as a cough syrup in cases of chronic tonsillitis and lung infection (Rood, 1994). Recent findings on its radical scavenging activity and antimutagenic activity could fulfil requirements for honeybush tea to be regarded as a functional food or nutraceutical (Hubbe, 2000; Marnewick *et al.*, 2000). It could even be considered superior to the traditionally drunk *Camellia sinensis* teas due to the low tannin content (Terblanche, 1982) and lack of caffeine (Greenish, 1881).

Phenolic compounds

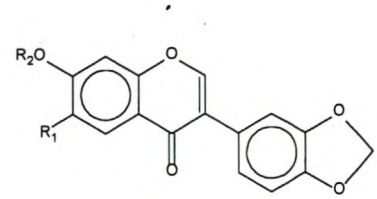
Fermented *C. intermedia* contains a wide variety of phenolic compounds (Figure 6) ranging from mangiferin, isomangiferin, hesperetin, hesperidin, eriodictyol and formononetin to small amounts of luteolin (Kamara, 1999; Ferreira *et al.,* 1998). Some of these compounds have been shown to be strong antimutagens as in the case of luteolin (Nakasugi, 2000) or to have antioxidant activity in the form of radical scavenging abilities (Rice-Evans *et al.*, 1996).

Compounds isolated from fermented *C. intermedia* and unfermented *C. subternata* can be ranked according to their TEAC values as follows: epigallocatechin gallate > luteolin > naringenin > hesperetin > hesperidin (Rice-Evans *et al.*, 1996). The ability of these compounds to inhibit lipid peroxidation was in the order luteolin > eriodictyol > hesperetin > naringenin (Mora *et al.*, 1990). A comparative study by Hubbe (2000) on the antioxidant activity showed that, luteolin and eriodictyol were the most effective scavengers of $O_2^{-\bullet}$, with activity comparable to that of the antioxidant enzyme superoxide dismutase (SOD), whereas hesperidin was the least effective. Comparison on a molar basis revealed that luteolin, mangiferin and isomangiferin were the most effective scavengers of the DPPH$^\bullet$ radical, with hesperidin again showing the weakest activity (Hubbe, 2000). Luteolin (97.1% inhibition) and the rooibos flavone orientin (97.7% inhibition) were also shown to be the most potent compounds, comparable to known synthetic antioxidants such as propyl-gallate in the DPPH$^\bullet$ assay (Mora *et al.*, 1990). Mangiferin rapidly scavenges the DPPH radical and inhibit both enzymatically and non-enzymatically induced lipid peroxidation (Sato *et al.*, 1992). For the inhibition of mutagenesis in the *Salmonella typhimurium* assay compounds identified from *Cyclopia* can be ranked in order of decreasing ability to inhibit mutagenesis: luteolin > hesperetin > naringenin against AFB_1 and hesperetin > naringenin > luteolin against MNNG (Choi *et al.*, 1994).

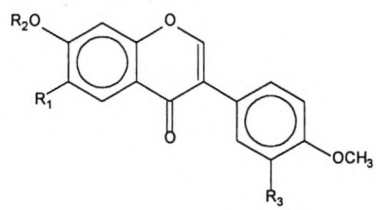
Apart from the antioxidant and antimutagenic properties of honeybush polyphenols other beneficial effects have been demonstrated for several of these compounds. The xanthone mangiferin is well known for its cardiotonic, spasmolitic, diuretic, antimicrobial and antiviral actions (Peres *et al.*, 2000; Simova *et al.*, 1986). The flavanone naringenin, has been shown to be much more effective than genistein the major soy isoflavone in its ability to inhibit proliferation of human breast cancer cells in culture. Genistein shares a close similarity in structure to naringenin (Carroll *et al.*, 1998). Genistein has been implicated in a variety of biological activities including allelopathic, estrogenic or proestrogenic, antihaemolytic, antioxidant and anticancer activities, as well as the inhibition of several enzymes, including catechol *O*-methyltransferase, DOPA decarboxylase, dopamin β-hydroxylase, histidine decarboxylase and lipase (Tahara & Ibrahim, 1995). Fermented *C. intermedia* has been shown to contain isoflavones such as pseudobaptigen, fujikinetin, formononetin, afformosin and calycosin (Ferreira *et al.*, 1998). The isoflavones are naturally occurring plant components that are structurally similar to the mammalian oestrogen oestrodiol-17β and exhibit oestrogenicity. These isoflavones or phytoestrogens share several



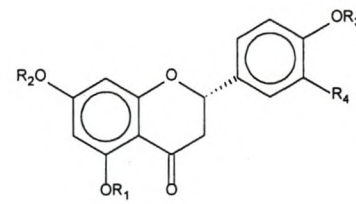
Flavone
Luteolin
R^1=H



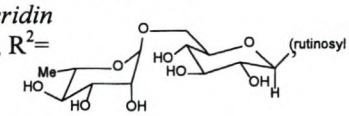
Isoflavones
Pseudobaptigen
R^1=R^2=H
Fujikinetin
R^1=OMe, R^2=H



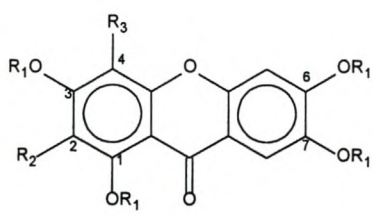
Isoflavones
Formononetin
R^1=R^2=R^3=H
Afrormosin
R^1=OMe, R^2=R^3=H
Calycosin
R^1=R^2=H, R^3=OH



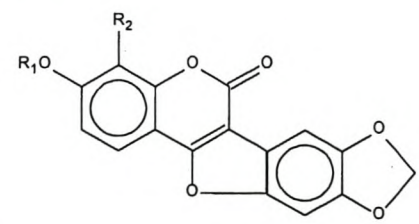
Flavanones
Naringenin
R^1=R^2=R^3=R^4=H
Eriodictyol
R^1=R^2=R^3=H, R^4=OH
Hesperetin
R^1=R^2=H, R^3=Me, R^4=OH
Hesperidin
R^1=H, R^2=



R^3=Me, R^4=OH



Xanthones
Mangiferin
R^1=R^3=H, R^2=2-β-D-glucopyranosyl
Isomangiferin
R^1=R^2=H, R^3=2-β-D-glucopyranosyl



Coumestans
Medicagol
R^1=R^2=H
Flemichapparin
R^1=Me, R^2=H
Sophoracoumestan
R^1=H, R^2=OMe

Figure 6. Structures of the major phenolic compounds from honeybush tea (Ferreira *et al.*, 1998).

features in common with oestrodiol, including a pair of hydroxyl groups separated by a similar distance and the presence of a phenolic ring which is prerequisite for binding to the oestrogen receptor (Cassidy *et al.*, 2000). Isoflavonoids do have properties in common with the flavonoids such as antioxidant, anticataract, anti-inflammatory and antiallergenic effects and they are also able to inhibit lipoxygenase activity. Hesperidin isolated from *C. intermedia* and *C. subternata* and eriocitrin present only in *C. subternata* inhibited the formation of 8-hydroxydeoxyguanosine, a product of deoxyguanosine that has undergone oxidation due to oxidative stress (Miyake *et al.*, 1998).

Studies on the antioxidant and antimutagenic activities of *Cyclopia*

The antioxidant and antimutagenic activities of *Cyclopia* have been studied previously by Hubbe (2000) and Marnewick *et al.*, (2000). The study by Hubbe (2000) involved the quantification of the *in vitro* antioxidant and antiradical potential of different species of traditional and unprocessed honeybush tea. The potency of the aqueous *Cyclopia* extracts in inhibiting linoleic acid peroxidation and scavenging of the 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) and the biologically relevant superoxide anion ($O_2^{\bullet -}$) radicals differed among species, depending on the test system used (Hubbe, 2000). Fermentation of different species of *Cyclopia* lowered the antiradical efficiency towards $DPPH^{\bullet}$ and superoxide, but not the linoleic acid peroxidation inhibiting activity. Unfermented *C. sessiliflora* with the highest total polyphenol content was the most effective scavenger of both $DPPH^{\bullet}$ and superoxide. Aqueous extracts of fermented and unfermented *C. genistoides* showed the most effective inhibition of linoleic acid peroxidation. The study carried out by Marnewick *et al.*, (2000) compared the antimutagenic activity of fermented and unfermented *C. intermedia* in comparison with *Aspalathus linearis* (Rooibos tea) using a variety of mutagens in the *S. typhimurium* assay. Various direct and indirect mutagens and plating techniques were used, allowing more understanding into the mode of antimutagenic action shown by the aqueous extracts of *C. intermedia*. Antimutagenic activity was observed against metabolically activated mutagens, 2-acetylaminofluorene (2-AAF) and aflatoxin B_1 (AFB_1) using tester strains TA98 and TA100. *Cyclopia intermedia* had no significant antimutagenic activity against the direct acting or oxidative mutagens tested, namely cumolhydroperoxide (CHP), hydrogen peroxide (H_2O_2) and methyl methanesulfonate (MMS). The strain TA102, designed to detect oxidative mutagens, was used with the latter mutagens. This lack of activity introduces new perspectives regarding the role of the polyphenolic compounds

known to exhibit antioxidant properties, in the protection against mutagenesis in the *Salmonella* assay. The mechanism behind the strong antimutagenic effect observed towards 2-AAF and AFB_1 was thought to be due to the polyphenols acting as electron acceptors to NADPH thus impeding the flow of electrons to cytochrome P_{450} and preventing the formation of genotoxic intermediates (Marnewick *et al.*, 2000).

5. CHARACTERISATION OF SOME OF THE BIOLOGICAL PROPERTIES OF *CYCLOPIA* SPECIES

A variety of *in vitro* methods can be employed for the study of the mechanisms involved in the degenerative diseases mentioned earlier namely those associated with lipid peroxidation and mutagenesis. The use of these *in vitro* methods for the elucidation of the antioxidant and antimutagenic actions against the effects of lipid peroxidation and mutagenesis, respectively can give an indication of the type of activity as well as the mode of action of a particular compound. It must, however be borne in mind that this does not predict what its actions will be *in vivo*.

Quantification of phenolic compounds

There are a variety of spectrophotometric and colorimetric methods that can be used for the quantification of various phenolic groups. Since the total polyphenolic content and the flavanol content are of interest for this research project the principles behind the methodology used for their quantification will be discussed.

Total polyphenols

Quantitative analysis of phenolic compounds in biological extracts can be carried out in many different ways. The most reliable method for the determination of total polyphenols is based on an oxidation reaction with the Folin-Ciocalteu reagent containing sodium phosphomolybdate and sodium tungstate (Marshall, 1992). The intensity of the resulting blue complex can be estimated with a colorimeter or with a spectrophotometer (λ_{max} at 725 nm). Compared to other methods, analyses of the Folin-Ciocalteu type are convenient, simple, require only commonly available equipment, and have produced a large body of comparable data according to Singleton & Rossi (1965). Under proper conditions the assay is inclusive

of monophenols and gives predictable (but variable by reactive groups per molecule) reactions with the types of phenols found in nature. Because different phenols react to different degrees, expression of the results as a single number such as milligrams per litre gallic acid equivalents is necessarily arbitrary. The reaction is also independent, quantitative, and predictable therefore the analysis of a mixture of phenols can be recalculated in terms of any other standard (Singleton & Rossi, 1965).

Flavanols

The total flavanol content of plant tissue is often estimated by the use of reagents containing aromatic aldehydes. The reaction of aldehydes with flavanols is well known with the substitution by aromatic aldehydes in acid solution resulting in coloured compounds that can be measured colorimetrically (Treutter *et al.*, 1994). Depending on the aldehyde the colours range from red to blue, corresponding to maximal absorbance at 510 nm and 640 nm after reaction with vanillin and dimethylaminocinnamaldehyde (DAC), respectively. However, the aldehyde reaction is not specific for flavanols. A wide range of secondary compounds can be substituted to form coloured products, but since most of the reaction products show different absorbance spectra, only a few have the chance to interfere with the DAC assay at 640 nm. Additionally, the relative sensitivity of the various substrates for the aldehyde is very low compared to other flavanols. Therefore, one would need a hundred or even a thousand fold greater concentration to reach the absorbance obtained by the flavanol-DAC-adduct (Treutter *et al.*, 1994). McMurrough & McDowell (1978) showed that the DAC reagent to has a sensitivity response similar to vanillin. They also showed that it was extremely reproducible over several years of use with different batches of DAC. The specificity of the DAC reagent was investigated for a range of phenolic compounds, many of which are known to occur in plants. No response was detected when the test was performed on numerous hydroxybenzoic and hydroxycinnamic acids, chlorogenic acid, phenol, *o*-catechol, rutin, hesperetin, hesperidin, quercetin, dihydroxyquercetin, myrecitin, phloridzin, naringenin, and naringin. Weak responses were obtained with resorcinol, orcinol, naphtoresorcinol, and phloretin and colour development was slow. Flavonoids with free *meta*-oriented hydroxy groups in the A ring and a single bond at the 2,3-position are capable of reacting with DAC. A much lower colour yield obtained with procyanindin B_3 suggests that at least one of the aromatic A-rings in this dimer is involved in intermolecular linkage and is therefore not available for reaction with DAC (McMurrough & McDowell, 1978).

Individual phenolic compounds

Thin-layer chromatography has been widely employed for polyphenols, because it is a highly effective, convenient and inexpensive technique. Individual phenolic compounds have been determined quantitatively by paper chromatography and semi-quantitatively by densitometric analysis of the coloured spots obtained by spraying a two-dimensional chromatogram with a suitable reagent (Marshall, 1992). Today it is also possible to separate and quantitate the individual polyphenols by means of high-performance liquid chromatography (HPLC) with great success (Marshall, 1992) and computer-controlled HPLC has become the analytical method of choice (Merken & Beecher, 2000). HPLC has advantages over other chromatographic and spectrophotometric procedures such as sensitivity, speed and ease of use. However, before using this procedure the polyphenols should first be fractionated into several chemical groups to separate the individual polyphenols effectively (Marshall, 1992). A reference standard is also necessary in order to quantify a specific compound. Certain reference standards may not always be readily available or only available at great costs. This method however is superior to the Folin-Ciocalteu method when reference compounds are available, since it does not give results relative to a single compound that is representative of all the phenolic compounds present.

Column conditions for the determination of flavonoids are almost exclusively reversed-phase. Elution systems are usually binary, with an aqueous acidified polar solvent such as aqueous acetic acid, perchloric acid, phosphoric acid, or formic acid and a less polar organic solvent such as methanol or acetonitrile, possibly acidified. Runs are generally an hour maximum, with equilibration between runs and flow rates are usually 1.0 or 1.5 mL min^{-1}. Thermostatically controlled columns are normally kept at ambient or slightly above ambient temperatures and injections generally range from 1 to 100 μL (Merken & Beecher, 2000).

Detection of the flavonoids is in the ultra-violet region since phenols absorb in this region. Two absorption bands are characteristic of flavonoids. Band II, with a maximum in the 240-285 nm range, is believed to arise from the A-ring. Band I with a maximum in the 300-550 nm range, presumably arises from the B-ring (Mabry *et al.*, 1970; Robards & Antolovic, 1997). Flavanones and their glycosides are generally detected at 280 nm (Krause & Galensa, 1991) and 290 nm (Bogdanov, 1989), while flavones, flavonols and flavonol glycosides are usually detected at wavelengths of 270 nm (Brolis *et al.*, 1998), 365 nm

(Crozier *et al.*, 1997) and 370 nm (Ewald *et al.*, 1999), although detection at 280 nm and 350 nm has been used (De Cooman *et al.*, 1998).

***In vitro* antioxidant methodology**

Tea and tea flavonoids have consistently demonstrated strong *in vitro* scavenging ability against numerous physiologically significant reactive oxygen species (ROS) (Wiseman *et al.*, 1997). Antioxidant activity is commonly tested on radicals such as the superoxide radical anion, singlet oxygen, hydroxyl radical, peroxyl radical and stable synthetic free radicals such as the radical cation of the compound 2,2,'-azinobis-(3-ethylbenzthiazoline)-6-sulfonic acid ($ABTS^{\bullet+}$) and the diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) (Wiseman *et al.*, 1997).

In testing putative antioxidant activity it is important to use biologically relevant ROS and sources generating such species. A relevant species that is known to be formed *in vivo* is $O_2^{\bullet-}$. It is largely, if not completely, converted by superoxide dismutase (SOD) or non-enzymatic dismutation into H_2O_2 (Halliwell, 1995). Unlike $O_2^{\bullet-}$, H_2O_2 is able to cross biological membranes, but both can find some targets within cells at which they can do direct damage. Only a few compounds, other than specific enzymes such as SOD and catalase, are able to remove $O_2^{\bullet-}$ and H_2O_2 at rapid rates. For example many thiols react with H_2O_2 and with $O_2^{\bullet-}$, but the rate constants for these reactions are low (usually $<103 M^{-1}.s^{-1}$) (Halliwell, 1995). Very high thiol concentrations (often >1 mM) would be required to achieve significant scavenging. Rate constants for the reaction of the superoxide radical with certain flavonoids, i.e. hesperidin, hesperetin and quercetin suggests valuable antioxidant activity in terms of the removal of $O_2^{\bullet-}$ and H_2O_2 from the cell (hesperidin = $2.8 \times 10^4 M^{-1} s^{-1}$, hesperetin = $5.9 \times 10^3 M^{-1} s^{-1}$, quercetin = $4.7 \times 10^4 M^{-1} s^{-1}$) (Halliwell & Gutteridge, 1989).

Much of the damage done by $O_2^{\bullet-}$ and H_2O_2 *in vivo* is thought to be due to their conversion into much more reactive species such as the $OH^{\bullet}$. The hydroxyl radical is highly reactive and is able to combine with almost all molecules found in living cells, with rate constants of 10^9-$10^{10} M^{-1} s^{-1}$ (Halliwell & Gutteridge, 1989). Thus almost everything in a cell is an $OH^{\bullet}$ scavenger. Attack of $OH^{\bullet}$ upon biological molecules yields a radical in all cases. In many cases carbon centred radicals are produced that can react with O_2 to give peroxyl radicals, e.g. (Halliwell, 1995).

$$R\text{-}H \xrightarrow{\cdot OH} H_2O + R^{\cdot} \xrightarrow{O_2} RO_2^{\cdot}$$

Good antioxidants should usually be effective as a hydrogen, electron or radical donor. Antioxidants acting to interfere with damage caused by $OH^{\bullet}$ *in vivo* will most likely not act by direct $OH^{\bullet}$ scavenging, but by scavenging H_2O_2, blocking formation of $O_2^{\bullet-}$ and/or H_2O_2 or by binding the transition metal ions needed for $OH^{\bullet}$ formation from $O_2^{\bullet-}$ and H_2O_2 (Halliwell & Gutteridge, 1989).

Many plant phenolics (especially flavonoids) have been styled as "antioxidants" because they inhibit lipid peroxidation – hence the appearance of "bioflavonoids" on the shelves of health-food stores. However, several plant phenolics can accelerate oxidative damage to non-lipid biomolecules such as DNA *in vitro*. They can do this by reducing metal ions and/or by oxidising to produce $O_2^{\bullet-}$ and H_2O_2. Thus an antioxidant in one system is not an antioxidant in all systems, and this must be borne in mind when evaluating 'natural' antioxidants ("natural" does not equate to "safe") (Halliwell, 1995).

Quantification of total antioxidant activity

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids, food extracts and pure compounds (Miller & Rice-Evans, 1997). Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range. Two types of approach have been taken. The first approach are the inhibition assays (Miller & Rice-Evans, 1997), in that the extent of the scavenging by hydrogen- or electron-donation of a pre-formed free radical, relative to that of a standard antioxidant compound (usually Trolox), is the marker of antioxidant activity. The second approach involves assays that require the addition of an antioxidant to a system generating the radical (Miller & Rice-Evans, 1997). The simple scavenging or inhibition assays, such as the TRAP (total reactive antioxidant potential or total radical-trapping antioxidant parameter) and the TEAC (Trolox equivalent antioxidant capacity) assays, have gained popularity because they enable high throughput screening on potential antioxidant capacity (Van den Berg *et al.*, 1999). Such methods are used to assess antioxidant capacity of biological matrices, such as plasma, as well as single compounds, food components and food extracts. The measurement of antioxidant activity towards oxidants that are not present in biological samples has become a controversial issue among scientists of this field (Prior & Cao, 1999). Of the previously mentioned TRAP and TEAC assay only the former makes use of a biologically relevant radical, the peroxyl radical. Prior & Cao (1999) have stated that chemically a pro-oxidant is

an oxidant of pathological importance and therefore although an antioxidant may be referred to as a reductant, a reductant may not necessarily be referred to as a antioxidant. Therefore if the methods use pro-oxidants to measure a compound's reducing potential, these compounds may certainly be referred to as antioxidants. However, assays that make use of oxidants of chemical origin are not measuring a biologically defined antioxidant, but rather a chemically defined reductant. Assays such as these are indirect measures of antioxidant power and are useful for preliminary screening of compounds due to their simplicity and affordability.

The TEAC assay was reported first by Miller *et al.* (1993) and then modified by Re *et al.* (1999) and is based on the scavenging of a long-lived radical cation ($ABTS^{\bullet+}$). The generation of the ABTS radical cation involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate (Re *et al.*, 1999). It has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm, as well as the more commonly used maximum of 415 nm. Addition of antioxidants to the pre-formed ABTS radical cation reduces it to an extent and on a time scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus the extent of decolourisation as a percentage inhibition of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox, a synthetic Vitamin E analogue, as a standard, under the same conditions (Re *et al.*, 1999). The method is applicable to study both water-soluble and lipid-soluble antioxidants, pure compounds and food extracts (Re *et al.*, 1999).

Another method whereby antioxidant power is referred to analogously as reducing ability is a redox linked colorimetric assay that measures the direct reducing ability of the antioxidant with regard to Fe^{3+} (Benzie & Strain, 1996). This method known as the FRAP (Ferric Reducing Antioxidant Power) assay measures the reduction of a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex at low pH to the intense blue ferrous (Fe^{2+}) form with an absorption maximum at 593 nm (Benzie & Strain, 1996). The reaction is non-specific and any half-reaction which has a less-positive redox potential, under reaction conditions, than the Fe^{3+}/Fe^{2+}-TPTZ half reaction will drive Fe^{3+}-TPTZ reduction (Benzie & Strain, 1996). In the FRAP assay excess Fe^{3+} is used, and the rate limiting factor of Fe^{2+}-TPTZ, and hence colour formation is the reducing ability of the sample (Benzie & Strain, 1996). This assay has been regarded as a direct test of "total antioxidant power" implying that there is no difference between a chemically defined reductant and a biologically defined antioxidant. What this assay really measures is the ability of a compound to reduce Fe^{3+} to produce Fe^{2+}.

It is well known that Fe^{2+} are pro-oxidants that can react with H_2O_2 to produce $OH^{\bullet}$, the most harmful free radical found *in vivo*. Why then can compounds that produce Fe^{2+} from Fe^{3+} be considered as antioxidants in the FRAP assay? The answer is probably that some antioxidants, such as ascorbic acid and uric acid, can reduce both reactive species and Fe^{3+}, and their ability in reducing Fe^{3+} may reflect their ability in reducing reactive species (Prior & Cao, 1999). But, not all reductants that are able to reduce Fe^{3+} are antioxidants and in addition an antioxidant that can effectively reduce pro-oxidants may not be able to efficiently reduce Fe^{3+}. For example, the FRAP assay does not measure the antioxidant activity of GSH, an important antioxidant *in vivo* (Prior & Cao, 1999).

Determination of ability to inhibit lipid peroxidation

Systems used for the study of oxidative damage to membranes include the use of erythrocytes, liver, heart or brain microsomes and artificially generated liposomes. Erythrocytes are often used in human studies investigating dietary influences on membrane susceptibility to oxidative damage (Wiseman, 1995). Microsomes are a heterogeneous mixture of vesicles derived from both endoplasmic reticulum and plasma membranes and are used as an *in vitro* test system to assess the ability of a wide range of drugs and dietary components to protect against membrane peroxidation (Wiseman, 1995). Liposomes, however, are an artificial lipid membrane used extensively as a model membrane system for studying the influence of dietary components and drugs on membrane peroxidation *in vitro* and are made by shaking or sonicating phospholipids in aqueous suspension (Wiseman, 1995).

Biological membranes are often rich in unsaturated fatty acids and bathed in an oxygen-rich, metal containing fluid. Therefore it is not surprising that membrane lipids are susceptible to peroxidative attack (Beuge & Aust, 1978). Microsomes isolated from liver have been shown to catalyse an NADPH-dependent peroxidation of endogenous unsaturated fatty acids in the presence of ferric ions and metal chelators, such as ADP or pyrophosphates. Non-enzymatic peroxidation of microsomal membranes also occurs and is probably mediated in part by endogenous hemoproteins and transition metals. Conditions that lead to the disruption of microsomal membranes, such as homogenisation and repeated freezing and thawing, enhance autocatalytic lipid peroxidation (Beuge & Aust, 1978). Other conditions that accelerate microsomal lipid peroxidation include exposure of the microsomes to γ-

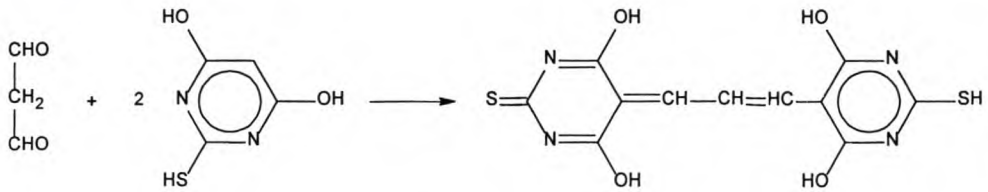
radiation, light, hyperbaric pressure, hyperoxia, ozone, nitrogen oxides, and radical initiators, such as dialuric acid (Beuge & Aust, 1978).

Since it is the peroxidation of LDL that concerns the degenerative diseases such as arteriosclerosis and coronary heart disease it is important to be aware of salient differences between liver microsomes, especially those isolated from species other than humans such as rats, and actual human LDL when testing antioxidants. Although the mechanism of lipid peroxidation in LDL is similar to that described for microsomal membranes, there are some important differences (Swanian & Ursini, 2000). The presence of an oxidation lag period in LDL that is negligible or very short in membranes such as microsomal or in artificial membranes is a very obvious difference. This distinction is evident when oxidation is initiated with iron or copper plus a reducing agent such as ascorbate. Using the well-known oxidising system consisting of complexed iron and ascorbate, a rapid peroxidation of microsomal lipids is seen without an apparent lag period. Biomembranes, and not LDL, exhibit an induction period for their oxidation. The duration of the induction period is in comparison with the large difference in the lag phase between biomembranes and LDL oxidation induced by copper or complexed-iron catalysts. Moreover, ascorbate plays an antioxidant role in LDL when present in micro-molar concentrations whereas in membranes antioxidant effect can be seen in the millimolar range. The reason for these difference may be ascribed to the nature of apolipoprotein B-100 (apo-B), its ability to bind and possibly reduce copper, interaction of apo-B with LDL lipids and the composition and physical order of LDL lipids (Swanian & Ursini, 2000).

There are various indirect methods for the measurement of lipid peroxidation. Products of lipid peroxidation such as conjugated dienes, lipid peroxides, thiobarbituric acid reactive substances [i.e. malondialdehyde (MDA)] and carbonyl compounds can be measured in a variety of ways using fluorescence, chemiluminescence or UV-visible spectrophotometric methods (Smith & Anderson, 1987; Wheatley, 2000). Contrary to shelf-life testing where the measurement of conjugated dienes appears to be the best index of lipid oxidizability, lipid peroxidation in biomedical research is commonly measured through thiobarbituric acid (TBA) reactive substances (TBARS) (Wiseman, 1995; Wheatley, 2000). It is well documented that the TBA test is not specific for MDA. A great variety of substances including sugars, amino acids, alkanals, alkenals and alkadienals other than MDA under appropriate conditions also form pink TBA complexes (Kosugi *et al.*, 1989; Esterbauer & Cheeseman, 1991). It would seem, however that using the protocol for peroxidized tissue sample such as microsomes there is little interference with other MDA precursors (protein-

MDA complexes or oxidised lipids) since these are removed by cold trichloroacetic acid (TCA) precipitation prior to the actual assay. Secondly, other TBA positive compounds, such as aldehydes, amino acids, sugars and fatty acid hydroperoxides, that could be present in the de-proteinized supernatant give only a very weak colour in the standard TBA assay (Esterbauer & Cheeseman, 1991).

In the TBA test reaction one molecule of MDA reacts with two molecules of TBA with the production of a red adduct (λ_{max} 532-535 nm and 245-305 nm) (Esterbauer & Cheeseman, 1991; Wheatley, 2000).



The reaction should be performed at pH 2-3 at 90-100° C for 10-15 minutes. The rate of reaction depends on the TBA concentration and on temperature. Using 80 mM TBA in the reaction mixture the reaction is complete in 5 min at 94°C, though 100°C for 30 min using 10 mM TBA is more typical. Typically the tissue sample (e.g. a liver microsomal suspension) is mixed with 2 volumes of cold 10% (w/v) TCA to precipitate the protein. The precipitate is pelleted by centrifugation and an aliquot of the supernatant is reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 minutes. The TBA reagent should be prepared as an aqueous solution and requires heating to dissolve the TBA solid (Esterbauer & Cheeseman, 1991). After cooling the absorbance is read at 532 nm and the concentration of MDA calculated based on the extinction coefficient (ε) value of 153 000. This value is an average of several slightly differing figures reported in the literature. The crystalline MDA-TBA adduct in water shows an absorption maximum at 532 nm (ε 159 000) (Esterbauer & Cheeseman, 1991).

To ensure that no further lipid oxidation occurs during the TBA assay, butylated hydroxytoluene (0.01 vol. % of a 2% BHT solution in ethanol) and ethylenediamine-tetraacetic acid disodium salt (EDTA) (1mM concentration) can be added to the sample prior to TCA precipitation (Esterbauer & Cheeseman, 1991).

In vitro measures of antimutagenicity

Identifying the chemicals that cause cancer in people is difficult due to a 5 to 30 year latent period between initial exposure to the carcinogen and the appearance of most types of human cancer (Newell, 1981). The identification of compounds that have the ability to inhibit cancer can be just as difficult. The development of bacterial systems for the detection of mutagenic properties of chemicals started in the early 1950's (Pott, 1775). Iyer & Szybalski (1958) introduced the plate assay technique. This method appeared to be suited for the rapid evaluation of potential mutagenic properties of large numbers of chemicals. A particular strain of *Escherichia coli* was used in these cases as the indicator organism. The majority of the mutagens identified in these studies were alkylating agents that were directly mutagenic. Gabridge & Legator (1969) developed a procedure in which a microbial indicator was incorporated in an animal system to identify pre-mutagenic agents because these bacterial systems tended to give false negatives for pre-mutagenic compounds that are converted into an active state in mammals. This procedure in which the activation of pre-mutagens is performed by the mammalian metabolism, is commonly specified as "host mediated assay". In 1973 Ames and co-workers introduced an *in vitro* test system for the screening of pre-mutagenic compounds. In the Ames assay the bioactivation process is mimicked by the addition of liver homogenate to the *S. typhimurium* strains (Maron & Ames, 1983; Bos, 1984).

Metabolic activation

Bioactivation or metabolic activation plays an important role when pre-mutagenic compounds are to be used in the *S. typhimurium* mutagenicity assay. Ideally, in terms of carcinogenicity screening, drug-metabolising enzyme preparations should be used in the *Salmonella* microsome assay that are able to produce all active metabolites formed in man *in vivo*. For practical reasons it is impossible to use fresh human tissue in mass screening. Animal tissue able to produce the same metabolites *in vitro* as those formed in man *in vivo* would be an acceptable alternative. However, no such animal tissue is available. As a rule rat liver S9 (9000 g supernatant) is incorporated in the test system (Bos, 1984; Maron & Ames, 1983). Rat liver homogenate from Aroclor-induced animals is widely used since the induction by Aroclor improves the metabolising capacity of the S9 for a variety of classes of compound. In general activation improves with increasing levels of S9 to an optimum and then declines, often dramatically. It has been shown that for activation of 2-AAF there is an optimal level of rat liver S9 fraction that is not markedly affected by the dose of 2-AAF used,

nor by the ratio of S9 to bacteria, nor by the presence of soft agar. It appears that diffusion of non-mutagenic metabolites of 2-AAF from the Ames top agar may be responsible for the difference in S9 optima and not the diffusion of co-factors NADP and glucose-6-phosphate or soluble S9 constituents as was previously thought (Forster *et al.*, 1980).

The Ames S. typhimurium mutagenicity assay

The "Ames test" is an assay used to indicate the mutagenicity of a compound using a set of histidine-requiring strains of *Salmonella typhimurium* (Maron & Ames, 1983). This test measures the rate of reversion of histidine auxotrophs to prototrophy in both the presence and absence of the chemical being tested (Maron & Ames, 1983). If the chemical is mutagenic it will cause changes or mutations in the DNA and thus increase the reversion rate. This test is not only able to identify possible mutagens but it is also capable of indicating the potency of the mutagen by the number of revertants (the more revertants the higher the potency). This test is based on the premise that mutagenesis and cancer induction both result from alteration of the DNA of a cell (Maron & Ames, 1983).

The Ames test was traditionally designed to assess the mutagenicity of certain test compounds but it has more recently been used to test the antimutagenicity of a specific compound (Maron & Ames, 1983). This is achieved by the addition of a known mutagen as well as the compound to be tested for antimutagenic activity. These compounds together with the bacterial tester strain and S9 (if the mutagen requires metabolic activation) are added to a soft top agar that is then poured onto minimal glucose agar containing a small amount of histidine and biotin (Maron & Ames, 1983). The addition of histidine to the minimal glucose agar allows the bacterial tester strain cells to undergo cell division until a background lawn is visible. The background lawn is important to determine bacterial toxicity since colonies appearing on a plate that has no background lawn are not revertants and should not be scored. These colonies arise from the surviving bacteria that live off the histidine present in the top agar. In testing a compound for antimutagenic activity a decrease in the reversion rate, when compared to a control plate containing only the mutagen, is desired.

The histidine auxotrophs of *S. typhimurium* are known as the tester strains and each of these tester strains has a specific mutation in one of the structural genes for histidine biosynthesis, including base-pair substitution and frameshift mutations (Maron & Ames, 1983). One mutation (*rfa*) causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules such as B[*a*]P that do not penetrate the normal cell wall. The other mutation (*uvrB*) is a deletion of a gene coding

for the DNA excision repair system, resulting in a greatly increased sensitivity in detecting many mutagens. For a technical reason the deletion of this gene has caused the deletion of the *bio* gene resulting in the bacteria requiring biotin as well as histidine to grow. An R-factor plasmid has also been included into some of the tester strains to make them able to detect mutagens that are only weakly detected or not at all in non R-factor parent strains. The genotypes of the TA strains used for mutagenesis testing can be seen in Table 3 (Maron & Ames, 1983).

When determining the reversion rate in the presence of a particular compound it is important to take into account spontaneous reversion of tester strains to their wild type (Maron & Ames, 1983). Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. There is, however, variability in the number of spontaneous revertants from one experiment to another, and therefore it is necessary to include sufficient replicates and controls that indicate spontaneous revertant numbers (Maron & Ames, 1983).

The Ames test can not only be used to measure the antimutagenic activity but it also gives an indication of the specific type of antimutagen. Desmutagenic, antimutagenic and bio-antimutagens can be distinguished from each other by altering the order of addition of the compounds to the reaction volume and through various incubation steps (Nagasugi *et al.*, 2000). Desmutagenic compounds can be identified by pre-incubating the mutagen and sample before adding it to the culture, whereas bio-antimutagenic activity can be assessed by pre-incubating the cells and mutagen before adding them to the sample. In determining only general antimutagenic activity everything is incubated together.

Bio-activity guided isolation of flavonoid rich extracts from *Cyclopia*

Owing to the complex nature of the phenolic fraction of *Cyclopia* species (Ferreira *et al.*, 1998; Kamara, 1999) fractionation of the plant extract is necessary in order to identify fractions that possess either high antioxidant and/or antimutagenic activity. Over the past decade, the techniques used for the isolation of flavonoids from plant material have not changed drastically. Solvent extraction, crystallisation, and column and paper chromatography are still very much in use today. Thin-layer chromatography, sephadex gel chromatography and gas-liquid chromatography are the major techniques still used (Hostettmann *et al.*, 1998).

Table 3. Genotypes of the TA strains used for mutagenesis testing (Maron & Ames, 1983).

Histidine mutation				LPS	Repair	R-factor
hisD6610 his0.1242 =TA88	hisD3052	hisG46	hisG428 (pAQ1)			
TA90	TA1538	TA1535	-	*rfa*	*uvrB*	-R
[TA97]	[TA98]	[TA100]	-	*rfa*	*uvrB*	+R
-	TA1978	TA1975	-	*rfa*	+	-R
TA110	TA94	TA92	-	+	+	+R
-	TA1534	TA1950	-	+	*uvrB*	-R
-	-	TA2410	-	+	*uvrB*	+R
TA89	TA1964	TA1530	-	Δ*gal*	*uvrB*	-R
-	TA2641	TA2631	-	Δ*gal*	*uvrB*	+R
-	-	-	[TA102]	*rfa*	+	+R

Tester strains in brackets are recommended for general mutagenesis testing. All strains were originally derived from *S. typhimurium* LT2. Wild-type genes are indicated by a +.

Plant preparation, extraction and preliminary purification

'like dissolves like' (Harborne, 1998)

Flavonoids occur in virtually all parts of the plant i.e. the root, heartwood, sapwood, bark, leaf, fruit and flower (Houghton & Raman, 1998). The method of isolation depends to some extent both on the source material and the type of flavonoid being isolated. Plant material is usually ground up or macerated before extraction. Pre-drying of plant material generally appears to increase the yield of extractives, possibly due to rupture of the cell structure and better solvent access. Solvents used for extraction are chosen according to the polarity of the flavonoids being studied (Markham, 1975). The less polar solvents are particularly useful for the extraction of flavonoid aglycones, whilst the more polar solvents are used if flavonoid glycosides are sought. The less polar aglycones such as isoflavones, flavanones and dihydroflavanols or flavones and flavonols which are highly methylated are usually extracted with solvents such as benzene, chloroform, ether or ethyl acetate (Markham, 1975). Flavonoid glycosides and the more polar aglycones such as the hydroxylated flavones, flavonols, biflavonoids, aurones and chalcones are generally isolated from plant material by extraction with acetone, alcohol, water or a combination of these (Markham, 1975).

Preliminary purification

When flavonoids of varying types are to be extracted from a single batch of plant material, a worthwhile method for preliminary separation is sequential solvent extraction with a number of solvents of varying polarity (Moure *et al.*, 2001). This can lead to separation of glycosides from aglycones and to the separation of polar from non-polar aglycones. Alternatively, the sequential solvent extraction of a crude extract may be used to produce the same type of separation (Moure *et al.*, 2001).

Precipitation with lead acetate has been widely used in the past as a method of isolating phenolics (especially those with *o*-dihydroxyl groups) from other extractives in crude extracts (Houghton & Raman, 1998). It has the disadvantage, however, that it does not precipitate certain phenols and may co-precipitate other compounds. A method using polyvinylpyrrolidone (PVP) as precipitant has been suggested for use in cases where lead acetate is unsatisfactory (Markham, 1975).

Column chromatography

Column chromatography remains the single most useful technique for the isolation of large quantities of flavonoids from crude plant extracts (Markham, 1975; Houghton & Raman,

1998). The advent of the chromatographic media, polyamide and Sephadex, has had a dramatic effect on the type and efficiency of separations achieved, and dry column techniques also seem to offer prospects of markedly improved chromatographic separations (Markham, 1975). Adsorbents commonly used for the separation of flavonoids include silica gel, kieselguhr, magnesol, cellulose, alumina, polyamide, Sephadex, C_{18} and ion exchange resins (Markham, 1975).

Polymeric supports

A preliminary passage of a polar sample over a polymeric support is an excellent means of removing unwanted hydrophilic contaminants such as amino-acids and carbohydrates (Hostettmann *et al.*, 1998). This is an approach employed by many Japanese groups for the pre-purification of saponins and other plant glycosides. The procedure typically involves chromatography on a highly porous polymer such as Dianion HP-20 or for the purification of polar compounds, Amberlite XAD-2 is used. This step may then be followed by open column chromatography on silica gel and/or sephadex LH-20 or reversed-phase C_{18} low pressure chromatography (Hostettmann *et al.*, 1998).

Size exclusion chromatography using sephadex LH-20

Sephadex is a highly cross-linked dextran on which separations are ideally obtained on the basis of molecular size (Markham, 1975). Thus substances are eluted in order of decreasing size. The gel must be swollen in water prior to use and the extent to which individual gels swell determines the molecular weight range of compounds which can be separated on that gel (Markham, 1975). Solvents other than water generally swell the gel to a lesser extent and compounds other than dextran may have different affinities for the gel, thus introducing effects due to adsorption. The hydroxypropylated dextran gel, Sephadex LH-20, is designed for use with organic solvents and the exclusion limit for this gel is normally considered to lie between MW 2000 and 10 000. Sephadex LH-20 gel filtration is not only an efficient preliminary fractionation step but it can also be employed as the very last step in isolation work, to remove last traces of solid material, salts or other extraneous matter (Hostettmann *et al.*, 1998). One of the reasons for its popularity at this stage of an isolation procedure (when amounts of pure product are often rather small) is probably that it causes minimal material losses (Hostettmann *et al.*, 1998).

Silica based materials

Silica gel is a useful adsorbent for the separation of flavonoids of quite a wide range of polarities. It has traditionally been used for the separation of isoflavones, flavanones, dihydroflavonols and highly methylated (acetylated) flavones and flavonols (Markham, 1975). In general terms, this range may be extended to include many of the more polar flavonoids simply by deactivation through the addition of water (Markham, 1975). In fact many of the variable chromatographic properties observed with silica adsorbents from different sources are undoubtedly attributable to the water content of the gel. The majority of preparative LC separations are still carried out in unmodified silica gel, mainly due to its lower cost, application and broad range of solvents as eluents, easy removal of solvents after fractionation and high flow rates. However, bonded phases (especially reversed-phase) are increasingly being used, these leading to a diminished risk of sample decomposition and less irreversible adsorption, among other advantages (Hostettmann *et al.*, 1998).

Highly polar and/or water soluble compounds are most conveniently separated by reversed-phase columns. Reversed-phase packing material is much more expensive than normal silica gel for use as a first open-column or flash purification step. However, there is less irreversible adsorption to derivatised silica gel and it can be regenerated. For this reason reversed-phase supports have been used in conjunction with semi-preparative HPLC (final purification) (Hostettmann *et al.*, 1998).

Thin-layer chromatography and visualisation

Screening of extracts can be done using thin-layer chromatography and different spray reagents. Apart from the anthocyanins and some of the more intensely coloured chalcones and aurones, flavonoids are not sufficiently coloured to be visible to the naked eye on a thin-layer plate thus some form of visualisation is necessary for spot detection. In many cases this is achieved by viewing the plate under UV light (366 nm) either in the presence or absence of ammonia vapour. The conjugated aromatic systems of flavonoids, show intense absorption bands in the UV and visible regions of the spectrum and those containing free phenolic groups undergo a characteristic and reversible colour change when fumed with ammonia vapour in ultra violet light (Harbourne, 1959). Some compounds, (e.g. flavans, isoflavones) can only be detected with chromogenic sprays of which the more frequently used are listed in Table 4.

Flavonoids in the same structural class often give characteristic colour reactions. For example, only flavanones form red magenta colours on reduction with sodium borohydride

Table 4. Effect of different chromogenic sprays on the colour of phenolic spots.

Compound	Structure	Colour reactions with			Colours in Ultraviolet		Reference
		$FeCl_3$	*Diazobenzene-sulphonic acid reagent*	*$NaBH_4/AlCl_3$*	*alone*	*with NH_3*	
Naringenin	4',5,7-trihydroxyflavanone	red-violet	orange	red			*Schmidtlein & Herrmann (1976)*
Eriodictyol	3',4',5,7-tetrahydroxyflavanone	blue-violet	red-brown	blue-violet			*Schmidtlein & Herrmann (1976)*
Hesperetin	3',5,7-trihydroxy-4'-methoxyflavanone	red-violet	red-brown	red			*Schmidtlein & Herrmann (1976)*
Hesperidin	3',5-dihydroxy-4'-methoxy-7-*O*-rutinosyl	yellow		Black			*Gage et al. (1951)*
Luteolin	3',4',5-trimethoxyflavone				lilac	orange-yellow	*Harborne (1959)*
Flavone					dull brown	bright green	*Harborne (1959)*
Flavonol					bright yellow	bright yellow	*Harborne (1959)*
Isoflavone					faint purple	faint purple	*Harborne (1959)*
Flavanone					colourless	colourless or faint yellow	*Harborne (1959)*

and subsequent exposure to acid vapour. In practice, it is not always possible to classify flavonoids according to their groups on the basis of their colour reactions alone. This is because some are exceptional in their behaviour. Some of the highly hydroxylated flavonols are dull brown rather than bright yellow in ultraviolet light. Other flavonols, i.e. those with hydroxyl groups in the 3', 4' and 5' positions such as myricetin, give a blue and not yellow colour after spraying with sodium carbonate solution (Harborne, 1959).

Screening for compounds with antioxidant activity in extracts can also be carried out using recently developed methods (Solar-Rivas *et al.*, 2000). An easy and fast test has been developed that is designed to compare the total free radical scavenging capacity of various compounds (Solar-Rivas *et al.*, 2000). Dilutions of extracts can be compared by spotting onto TLC silica gel layers in the form of a dot-blot test. The layers are stained with a 0.4 mM methanolic solution of the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$). One hour after staining, the bands containing antioxidative compounds can be seen as bright yellow on a pink-purple background (Solar-Rivas *et al.*, 2000).

6. CONCLUSION

The current awareness among consumers with regards to diet and health and delaying of ageing has fuelled the functional and nutraceutical markets. Natural antioxidants are ideal ingredients for inclusion into these products, because of the role they play in reducing oxidative stress associated with degenerative diseases such as coronary heart disease, atherosclerosis, cancer and acceleration of the ageing process. The safety aspects related to the inclusion of isolated plant flavonoids or flavonoid-rich extracts into functional foods are not yet fully understood making the regulation of such foods difficult. New information on the bioavailability of these compounds has shown that absorption of these compounds in their aglycone or glycosidic form may be far greater than was originally thought. Issues regarding contradictory evidence obtained from *in vitro* versus *in vivo* studies also make it difficult to forecast the relative safety of a compound for use in functional and nutraceutical products, ultimately demanding that these ingredients be treated in the same manner as pharmaceutical ingredients. However, for preliminary work and investigative studies, *in vitro* antioxidant and antimutagenicity assays are very useful and inexpensive to perform and can be extrapolated to *in vivo* studies. These assays can be useful to analytical laboratories for routine quality checks or screening of new compounds, extracts or products.

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CHAPTER 3

ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY OF FREEZE-DRIED AQUEOUS EXTRACTS OF *CYCLOPIA INTERMEDIA, C. SUBTERNATA, C. SESSILIFLORA* AND *C. GENISTOIDES* AND THE CONTRIBUTION MADE BY THE MAJOR PHENOLIC COMPOUNDS

ABSTRACT

Four commercially utilised species of *Cyclopia* (*C. intermedia, C. subternata, C. sessiliflora* and *C. genistoides*) were compared according to the *in vitro* antioxidant and antimutagenic activities of their aqueous extracts. Scavenging of the synthetic 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$), reduction of a ferric-tripyridyltriazine complex and inhibition of Fe^{2+} induced lipid peroxidation using rat liver microsomes were used as measures of antioxidant activity. Antimutagenic activity of aqueous extracts against metabolically activated 2-acetylaminofluorene (2-AAF) using tester strain TA98 was determined in the *Salmonella typhimurium* antimutagenicity (AMES) assay. Data obtained from the spectrophotometric quantification of the total polyphenols, flavanols and flavonols + flavones and HPLC quantification of the major phenolic compounds, mangiferin + isomangiferin and hesperidin, provided some interesting perspectives. Unfermented species yielded higher total soluble solids (27-39%), total polyphenol (27-33%), flavanol (0.7-2.4%) and flavonol + flavone (3.2-8.5%) contents than their fermented counterparts (16-35%, 16-22%, 0.1-0.6% and 2.6-7.6%, respectively). Antioxidant activity correlated with the total polyphenol ($r \geq 079$; $P < 0.0001$) and flavanol ($r = 0.53$; $P < 0.0001$) contents of the extracts. The flavonol + flavone content gave a negative correlation with antimutagenic activity ($r = -0.60$; $P < 0.0001$). The major phenolic compounds, mangiferin + isomangiferin and hesperidin, decreased substantially with fermentation. Mangiferin was the most effective $ABTS^{\bullet+}$ scavenger and ferric reducer, but gave moderate inhibition of lipid peroxidation and was not effective as an antimutagen. Hesperidin exhibited promutagenic activity.

Overall, unfermented *C. intermedia* and *C. sessiliflora* had the highest antioxidant activities, although *C. genistoides* contained significantly more mangiferin + isomangiferin (86% of the total polyphenol content as opposed to 39% for *C. sessiliflora*, 20% for *C.*

intermedia and 12% for *C. subternata*). *Cyclopia genistoides* was the most potent of the fermented plant material in all the antioxidant assays. Fermentation decreased the antimutagenicity of the aqueous extracts of the most potent species, *C. intermedia* and *C. sessiliflora*, but it had no effect on the antimutagenicity of *C. subternata*. The latter, together with *C. sessiliflora*, were the most potent of the fermented species. *Cyclopia genistoides* changed from promutagenic to antimutagenic with fermentation. This could partly attributed to the decrease in its flavonol + flavone content. Antioxidant activity measured in the three different assays correlated ($r \geq 0.79$; $P < 0.0001$) with each other, but no correlation with antimutagenic activity was demonstrated.

INTRODUCTION

Honeybush tea, an herbal beverage originating in South Africa, is prepared from *Cyclopia*, a fynbos plant indigenous to the Cape fynbos region. Currently four of the *c.* 24 species are utilised commercially namely, *C. intermedia* E. Mey., *C. subternata* Vogel, *C. sessiliflora* Eckl. & Zeyh. and *C. genistoides* (L.) R. Br. Although honeybush tea contains no caffeine (Greenish, 1881) and has a low tannin content (Terblanche, 1982), health promoting properties such as antioxidant and antimutagenic activities are increasingly important in marketing it as an herbal tea. Hubbe (2000) showed that considerable differences in antioxidant activity between species exist and that fermentation, an essential step for development of the characteristic flavour, reduces activity, partly as a result of a decrease in mangiferin content. This xanthone with moderate antioxidant activity (Hubbe, 2000) is one of the predominant monomeric polyphenols present in *C. intermedia*, *C. sessiliflora* and *C. genistoides* (Joubert *et al.*, 2002). Unfermented *C. sessiliflora* (with the highest total polyphenol content) was the most effective scavenger of both DPPH radical and superoxide anion radical, while fermented and unfermented *C. genistoides* gave the best protection against linoleic acid peroxidation (Hubbe, 2000). Up to date only the antimutagenic activity of unfermented and fermented *C. intermedia* has been studied (Marnewick *et al.*, 2000). Antimutagenic activity was observed against metabolically activated mutagens, but there was no significant effect against direct acting or oxidative mutagens.

Large differences in the phenolic composition have been found between *C. intermedia* and *C. subternata* with some of the compounds both antimutagens and antioxidants. Hesperetin, and especially luteolin, have excellent antimutagenic activity in the *S.*

typhimurium assay (Nakasugi *et al.*, 2000). Both displayed antioxidant activity in the $ABTS^{\bullet+}$ (Rice-Evans *et al.*, 1996) and $DPPH^{\bullet}$ assays (Mora *et al.*, 1990; Hubbe, 2000). Naringenin has moderate antioxidant activity in the $ABTS^{\bullet+}$ assay (Rice-Evans *et al.*, 1996) and excellent antimutagenic activity against aflatoxin B_1 in TA100 (Choi *et al.*, 1994). The highly effective radical scavenger, epigallocatechin 3-*O*-gallate (Zhao *et al.*, 1989) isolated from unfermented *C. subternata* (Brand, 2002), is associated with high bio-antimutagenic activity (Kada *et al.*, 1985).

The objective of this study was to extend current knowledge on the *in vitro* antioxidant and antimutagenic activities of the aqueous extracts of honeybush tea. For comparative purposes, extracts of both unfermented and fermented *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides* were investigated since "green" honeybush, the unfermented tea, has recently been commercialised. Colorimetric methods and HPLC analysis were used to quantify phenolic groups and individual compounds of extracts, respectively. Antioxidant activity was determined as the ability to scavenge the synthetic 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) radical cation, reduce a ferric-tripyridyltriazine complex and inhibit Fe(II) induced lipid peroxidation in a model membrane system, using rat liver microsomes. Antimutagenic activity was determined as the ability to inhibit 2-acetylaminofluorene induced mutagenesis of the tester strain TA98, in the presence of metabolic activation. Differences in activity between species were discussed in terms of phenolic composition and the activity of the major phenolic compounds.

MATERIALS AND METHODS

Chemicals

Isomangiferin, isolated from fermented plant material of *C. intermedia* (*ex* Langkloof), was kindly supplied by Prof. Daneel Ferreira, formerly of the Department of Chemistry, University of the Orange Free State, Bloemfontein, South Africa. Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthene-9-one), hesperetin (5,7,3'-dihydroxy-4'-methoxyflavanone), hesperidin (5,3'-dihydroxy-4'methoxy-7-O-rutinosylflavanone), naringenin (5,7,4'-trihydroxy-flavanone), gallic acid, potassium persulphate ($K_2S_2O_8$), butylated hydroxytoluene (BHT) (2,[6]-di-tert-butyl-*p*-cresol) and 2-acetylaminofluorene (2-AAF) of the highest available purity were purchased from Sigma Chemical Co. (St. Louis, United States of America). Eriodictyol (5,7,3',4'-tetrahydoxyflavanone), formononetin (7-hydroxy-4'-methoxyisoflavone)

and luteolin (5,7,3',4'-tetrahydroxyflavones) were purchased from Extrasynthese (Genay Cedex, France). 2,4,5-tri(2-pyridyl)-S-triazine (TPTZ) (99%) were supplied by Fluka (Poole, England). Folin Ciocalteau's phenol reagent, iron(II)sulphate heptahydrate ($FeSO_4 \bullet 7H_2O$) (99.5%) and iron(II)chloride tetrahydrate ($FeCl_2 \bullet 4H_2O$) (99%) were obtained from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97%) and thiobarbituric acid (TBA) (98%) were purchased from Aldrich (Steinheim, Germany) and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) from Boehringer Mannheim (Mannheim, Germany). All other chemicals were analytical grade. Deionised water refers to water treated with a Modulab Water Purification System (Separations, Cape Town). This consisted of a 5 μm filter to remove coarse particles followed sequentially by a carbon filter, a reverse osmosis process for the removal of >95% of ions, a mixed bed deioniser to obtain water at a conductivity level of 0.1 $\mu s.cm^{-1}$ and finally a filter (0.22 μm) for the removal of micro-organisms. For HPLC eluent and sample preparation, deionised water was further purified by means of a Milli-Q 185 Académic Plus water purification system (Millipore GmbH, Eschborn, Germany).

Spectrophotometric equipment

A GBC 911A UV/Visible spectrophotometer (BGC Scientific equipment (Pty.) Ltd., Dandenong, Victoria, Australia) and a 1 cm path length quartz cuvette were used for all spectrophotometric measurements.

Plant material

Six bundles (*c.* 5 kg per bundle) of *C. intermedia* were harvested in the Haarlem area in March 2000. Two bundles (*c.* 5 kg per bundle) of *C. subternata* were harvested from both 2- and 3-year-old plantations in Waboomskraal, as well as young (3 months) and old regrowth (12 months) from a 2-year-old plantation in Du Toitskloof. Seven bundles of *c.* 4 kg each of *C. genistoides* (West Coast type) and *C. sessiliflora* were harvested from plantations at Pearly Beach (March 2001, 5 year-old plantation, previous harvest June/July 2000) and Helderfontein, Stellenbosch (February 2001, 2.5-year-old plantation), respectively.

The harvested plant material was processed according to the standardised processing procedure of Du Toit & Joubert (1998) into fermented and unfermented counterparts. The leaves and stems of each of the species were cut into small pieces (≤ 4mm) using a modified fodder cutter. The chopped plant material (1200 g), moistened with deionised water (300 g)

and placed into four glass containers (320 g each), was allowed to undergo a chemical "fermentation" process (70°C for 60 h). The fermented material was then dried (40°C for 12 h) in a drying tunnel (Decon Humidifier, Continental Fan Works CC., Cape Town, South Africa). The unfermented material was prepared by drying the remaining material of each bundle directly after cutting in the drying tunnel (40°C for 12 h). The dried tea (< 10% moisture content; wet basis) was sieved with an Endecott test sieve (2 mm) (London, England). The fraction containing leaves and small pieces of stems (≤ 2 mm) was pulverised in a Retch rotary mill and stored in plastic containers in the dark.

Preparation of freeze-dried aqueous extracts

Aqueous extracts of the plant material were prepared by steeping 100 g of pulverised plant material in 1000 mL freshly boiled deionised water for 5 minutes. Extracts were then filtered through a Buchner filter using a 125 μm mesh cloth (Polymer PES D25/35 supplied by Swiss Silk Bolting Cloth Mfg. Co. Ltd, Zurich, Switzerland) to remove the majority of the plant material followed by filtration through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England) for removal of the finer particles. The filtrate was frozen (-20°C) in plastic trays (170 x 115 x 30 mm) before freeze-drying in an Atlas pilot-scale freeze-drier (Denmark model, Copenhagen, Denmark, 40°C shelf temperature). The freeze-dried soluble solids were stored in sealed clear glass vials in desiccators in the dark.

Determination of the total soluble solids, total polyphenol, flavanol and flavonol contents of aqueous extracts

The total soluble solids content of the aqueous extracts (g 100 mL^{-1}) was determined gravimetrically in duplicate on 20 mL aliquots. The aliquots were evaporated to dryness in nickel moisture dishes on a steam bath (Baird & Tatlock Ltd., Chadwell Heath, Essex, London), followed by drying under vacuum (70°C for 16 h).

Total polyphenol (TP) (Singleton & Rossi, 1965) and flavanol (McMurrough & McDowell, 1978) contents of the dry aqueous extract (DAE) were quantified colorimetrically in triplicate and expressed as g gallic acid equivalents 100 g^{-1} and g catechin equivalence 100 g^{-1} DAE, respectively. With absorption given by Band I (usually 300-380 nm) due to the B-ring cinnamoyl system of flavones and flavanols (Mabry *et* al., 1970) these compounds were estimated spectrophotometrically at 360 nm (Mazza *et al.*, 1999) and expressed as g quercetin equivalents 100 g^{-1} DAE. A stock solution of DAE (*c.* 1.2 mg mL^{-1}) was prepared for each

procedure (total polyphenols, flavanols and flavonols + flavones) by diluting with deionised water and ultra-sonicating for 5 min. The gallic acid equivalents of mangiferin and hesperidin were determined by reaction with the Folin-Ciocalteu reagent. The pure compounds, including the gallic acid, were diluted in DMSO to give a dilution series with absorbance readings of between 0.2 and 0.8 after reaction with the Folin Ciocalteu reagent.

HPLC analysis of aqueous extracts of unfermented and fermented species

Stock solutions (10 mg mL^{-1}) DAE in HPLC grade water were prepared through ultra-sonication for 5 min and filtration through a 13 mm 0.45 μm HV filter from Millipore (Ireland) for the quantification of mangiferin + isomangiferin and hesperidin. HPLC separations were performed on a Waters LC Module 1 Plus equipped with a UV-Vis detector and Millennium software from Microsep (South Africa). The DAE solution (10 μL) was injected in duplicate onto an Agilent Technologies Zorbax SB-C_{18} column (C_{18} reverse-phase, 3.5 μm particle size, 150 x 3 mm) purchased from Chemetrix (South Africa) with a Jour-Guard (C_{18} reverse-phase, 5 μm particle size, 7.5 x 4.6 mm) pre-column purchased from Separations (Cape Town, South Africa). The column temperature was maintained at 30°C. Separations were performed according to the acetic acid:acetonitrile solvent gradient program of Joubert *et al.* (2002). Peaks were tentatively identified according to retention times of mangiferin (for mangiferin + isomangiferin that co-eluted), hesperidin, luteolin, eriodictyol, formononetin, naringenin and hesperetin standards (see addendum A for chromatogram with retention times). Peak area used for calculation of the concentration of the compounds, was obtained through valley to valley integration. For the standard curves, concentration gradients of 0.3-9.0 μg 10 μL^{-1} of mangiferin (for mangiferin + isomangiferin) and 0.07-2.00 μg 10 μL^{-1} of hesperidin were prepared in DMSO and filtered through a 13 mm 0.45 μm filter. Samples were injected by means of an automatic injector and the absorbance measured at 280 nm.

Radical scavenging and ferric reducing abilities in the $ABTS^{\bullet+}$ and FRAP assays.

The radical scavenging activity of the aqueous extracts was determined using the radical cation 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonate) decolourisation assay according to the procedure of Re *et al.* (1999). Modifications made to the method for practical purposes involved the increase of the reaction temperature from 30°C to 37°C, the sample volume added to 3 mL $ABTS^{\bullet+}$ solution from 30 to 150 μL and the reaction time from 1 min to 4 min. A standard curve for Trolox (dissolved in ethanol) with a concentration range of 5-20 μM

(105-420 μM before addition to the reaction mixture of 3.15 mL) in the final reaction mixture was used. Controls consisted of ethanol and deionised water for the standard curve and samples, respectively. A stock solution of DAE was prepared (*c.* 1.2 mg mL^{-1}) in cold deionised water and dissolved with ultra-sonication for 5 min. The stock solution was diluted to give 20% and 80% scavenging of $ABTS^{\bullet+}$. The concentration of $ABTS^{\bullet+}$ remaining in the reaction volume at 734 nm was calculated as follows:

$$\text{Concentration (nmol)} = \frac{A\ V}{\varepsilon\ L\ 1000}$$

where:

A = absorbance at 734 nm

ε = extinction coefficient of $ABTS^{\bullet+}$ radical cation in ethanol (1.6×10^4 mol^{-1} cm^{-1}) (Re *et al.*, 1999)

L = path length (cm)

V = reaction volume (3.15 mL)

The concentration of scavenged $ABTS^{\bullet+}$ was calculated as the difference between the remaining $ABTS^{\bullet+}$ and that of the control. This value was plotted against the Trolox concentration (x-axis) in the reaction mixture (μmol) and the linear equation used to calculate the total antioxidant activity μmol Trolox equivalents g^{-1} DAE. Owing to the variation in the initial concentration of $ABTS^{\bullet+}$ from one experiment to another percentage inhibition as in Re *et al.* (1999) was not used to calculate Trolox equivalents. The percentage of the scavenged $ABTS^{\bullet+}$ will depend on the initial amount available, while the amount scavenged by the sample will remain the same thus ultimately affecting the Trolox equivalents calculated.

The ferric reducing abilities of the aqueous extracts were determined according to the ferric reducing antioxidant power assay (FRAP) set out by Benzie & Strain (1999). Iron(II)sulphate heptahydrate was used for the standard curve at a concentration range of 12.9-38.7 μM (400-1200 μM before addition to the reaction mixture). The stock solution of the DAE was prepared as for the $ABTS^{\bullet+}$ assay, and diluted in order to give a reaction mixture with an absorbance of between 0.2 and 0.8 at 593 nm. The reaction was carried out at 37°C for 4 min. The FRAP value of the DAE was expressed as μmol $FeSO_4 \bullet 7H_2O$ g^{-1} DAE.

The $ABTS^{\bullet+}$ and FRAP values obtained for the DAE were recalculated on a total polyphenol basis to obtain an indication of the total polyphenol potency. The $ABTS^{\bullet+}$ and

FRAP assays were also performed on the pure compounds, mangiferin, and hesperidin to calculate their apparent contribution to the $ABTS^{\bullet+}$ and FRAP values of the DAE. It was assumed that no synergistic or antagonistic effects between these compounds and others present in the DAE, existed. The pure compounds were dissolved in DMSO to a concentration of between 2.6-10.4 μM (mangiferin) and 6.2-31.2 μM (hesperidin) in the reaction mixture for the $ABTS^{\bullet+}$ assay and 5.6-45.1 μM (mangiferin) and 28.2-225.5 μM (hesperidin) for the FRAP assay.

Inhibition of microsomal lipid peroxidation in a model membrane assay

Inhibition of lipid peroxidation in rat liver microsomes by the aqueous extracts was determined according to a modified version of the method by Yen & Hsieh (1998). Rat liver microsomes were prepared according to the method by van Acker *et al.* (1996). Rat livers were excised from male Fischer rats weighing approximately 200 g. The livers were washed in 0.15 M KCl. Approximately 1 g liver to 3 mL 0.15 M KCl was homogenised with a Polytron at 37 000 rpm for *c.* 30 seconds. This was filtered through a double layer of cheesecloth before homogenising firstly in a loose and then a tight Dounce. The homogenate was centrifuged in a Sorvall Superspeed RC2-B centrifuge (Separations Scientific, South Africa) at 9 000 x g for 10 minutes at 4°C. Subsequently the supernatant was centrifuged at 100 000 x g for 60 minutes at 4°C in a Beckmann C8-70M Ultracentrifuge (Beckman Instruments, California). The supernatant was discarded and the microsomal pellet was washed with 50 mM phoshate buffer (pH 7.4) and centrifuged again at 100 000 x g for 60 minutes at 4°C. The microsomal pellet was suspended in phosphate buffer and stored in 1 ml Eppindorf tubes at -80°C. The protein concentration of the microsomal preparation was determined according to the method of Bradford (1976). For the microsomal lipid peroxidation assay modifications made to the method of Yen & Hsieh (1998) included the use of rat liver microsomes at a concentration of 1 mg mL^{-1} protein instead of 0.5mg mL^{-1} in the reaction volume and the omission of hydrogen peroxide. Aqueous extract (0.1 mL) and 2.5 mM $FeCl_2$ (0.2 mL) as initiator were added to the microsomes (1 mg mL^{-1}) in 13 mm x 120 mm disposable glass tubes (soaked in a 5% ethylene diamine tetra-acetic acid di-sodium salt (EDTA) solution, rinsed with deionised water and dried before use) to give a final reaction volume of 1 mL. A positive control, containing the microsomal mixture and $FeCl_2$, and a sample blank containing the extract, $FeCl_2$ and buffer, were included. The reaction mixtures were incubated at 37°C for 1 h. At the end of incubation 2 mL of a chilled mixture of EDTA

(1 mM) and BHT (0.01%) in 10% trichloroacetic acid was added to stop the reaction. The mixtures were centrifuged at 3600 rpm (1448 x g) in a Hettich Universal 16 centrifuge (Centrotec, Cape Town) for 25 minutes and the supernatant (2 mL) decanted into borosillicate screw top test tubes. Two millilitres of 0.67% TBA was added to the decanted supernatant before incubation at 90°C for 20 minutes. The reaction mixture was then allowed to cool in ice water for 5 minutes and stand at room temperature for a further 10 minutes. Absorption was read at 532 nm and the percentage inhibition calculated from the percentage malonaldehyde (MDA) formed in the presence of the extract relative to that formed in the positive control:

$$\text{MDA concentration (mol L}^{-1}) = \frac{A}{\varepsilon L}$$

where:

A = absorbance at 532 nm

ε = extinction coefficient of MDA (153 000 M^{-1} cm^{-1}) (Yen & Hsieh, 1998)

L = path length (cm)

$$\% \text{ Inhibition} = \frac{[MDA]_{control} - [MDA]_{sample}}{[MDA]_{control}}$$

Total polyphenol (TP) potency of the DAE polyphenols was calculated as the ratio of the percentage inhibition to TP. The concentration of the pure compounds, mangiferin and hesperidin, required to give a dose response of between 20% and 80% inhibition, was established.

Antimutagenic activity (*S. typhimurium* antimutagenicity assay)

The antimutagenic activity of the aqueous extracts was determined using the *S. typhimurium* antimutagenicity assay by Maron & Ames (1983). The *S. typhimurium* tester strain, TA98, was kindly provided by Dr. B.N. Ames (Berkely, CA). For the antimutagenicity assay 0.1 mL of the mutagen (5 μg 2-AAF $plate^{-1}$), 0.1 mL of DAE (1% and 2% for the unfermented and 2% and 5% for the fermented DAE), 0.5 mL S9-mix and 0.1 mL of an overnight bacterial culture per plate were added to 2 mL of molten top agar, containing 0.05 mM biotin-histidine and then dispersed onto minimal glucose agar (Vogel-Bonner medium E) plates. Vogel-Bonner medium E was prepared at a concentration of 25x and added to the minimal glucose agar plates. The

plates were allowed to set and then incubated in the dark at 37°C for 48 hours. Revertant colonies on the test plates and on the control plates were counted and the presence of a background lawn on all plates confirmed. Mammalian liver S9 was obtained from Aroclor 1254 induced male Fischer rats weighing approximately 200 g as described by Maron & Ames (1983). The S9 homogenate was incorporated into the S9-mixture at a level of 2 mg mL^{-1} protein as determined using the method of Bradford (1976). Control plates, containing S9-mix and TA98, were also included to obtain the background or spontaneous revertant colonies. The positive control plates contained S9-mix, 2-AAF and TA98 to determine the maximum number of revertants. Since 2-AAF is a mutagen requiring metabolic activation negative control plates containing 2-AAF and TA98 were included to guarantee that no reversion took place in the absence of the S9-mix.

Antimutagenic activity of the pure compounds, mangiferin and hesperidin dissolved in DMSO, was determined in a dose response manner. Extracts and compounds giving pro-mutagenic activity were tested for mutagenic activity in the absence and presence of S9.

Statistical analysis

One way analysis of variance followed by Student's t-LSD (SAS Release version 6.12) was performed on the means to determine significant differences between species (unfermented and fermented) at a significance level of 0.05. All the means were correlated and Pearson's correlation coefficient obtained.

RESULTS

Total soluble solids content and phenolic composition of freeze-dried aqueous extracts

The total soluble solids and its total polyphenol, flavanol and flavonol + flavone contents of the fermented and unfermented species of *Cyclopia*, summarised in Table 1, showed significant differences ($P < 0.05$) between some species depending on the polyphenol group. These values decreased significantly for all of the species after fermentation. *Cyclopia genistoides* showed the smallest percentage decrease in total soluble solids (10.5%), total polyphenol (24.4%), flavanol (52.8%) and flavonol + flavone (10.8%) contents of the DAE after fermentation. *Cyclopia subternata* had the greatest loss in total soluble solids (40.9%) and total polyphenols (47.1%) with fermentation. Of the unfermented plant material the DAE of *C. subternata* had the highest total polyphenol content, but for fermented tea, the DAE of *C.*

genistoides contained significantly more total polyphenols than that of the other species. The total polyphenol content of unfermented *C. intermedia*, *C. sessiliflora* and *C. genistoides* did not differ significantly, while after fermentation the total polyphenol content of *C. genistoides* differed significantly from *C. intermedia*, *C. subternata* and *C. sessiliflora*, that were not significantly different. The flavanol contents of the DAE both unfermented and fermented *Cyclopia* species were low in comparison to their total polyphenol content. *Cyclopia intermedia* had the highest flavanol content, but also the lowest flavonol + flavone content. This latter group was most abundant in *C. genistoides*.

The relative changes in phenolic composition that occur with fermentation can be seen from the typical chromatograms of the unfermented and fermented *Cyclopia* species in Figures 1 and 2. No separation of mangiferin and isomangiferin was achieved under the separating conditions used. Differences in the phenolic composition between species is apparent due to the presence of unknown peaks that could not be identified as one of the 7 compounds tested for (i.e. luteolin, naringenin, mangiferin, hesperidin, hesperetin, eriodictyol or formononetin). For example unfermented *C. intermedia* and *C. subternata* differed from the other species due to the unidentifiable peaks at retention times of *c.* 18.2 min and 11.4 min, respectively. These compounds are present in different ratios amongst the species. Mangiferin + isomangiferin contents of the unfermented *Cyclopia* species differed significantly (Table 2). The DAE of unfermented *C. genistoides* had the highest mangiferin + isomangiferin content at 11.69% while the level was as low as 1.69% for unfermented *C. subternata*. The levels decreased substantially with fermentation (Table 2). Approximately 40% of the mangiferin + isomangiferin in *C. genistoides* was retained after fermentation compared to only < 6% for *C. sessiliflora* and ≥ 11% for *C. intermedia* and *C. subternata*. After fermentation the mangiferin + isomangiferin content of *C. intermedia*, *C. subternata* and *C. sessiliflora* did not differ significantly, but fermented *C. genistoides* had more than 10 times the mangiferin + isomangiferin content of these species. The hesperidin content of unfermented *Cyclopia* was much lower than the mangiferin + isomangiferin content (Table 2). The highest level was obtained for unfermented *C. intermedia* (1.3%) followed by *C. genistoides* (1.05%). The hesperidin content of *C. subternata* and *C. sessiliflora* at 0.48 and 0.58%, respectively, was not significantly different ($P > 0.05$). Hesperidin was less affected by fermentation than mangiferin + isomangiferin, except for *C. genistoides* with a decrease of 64% in hesperidin content. Approximately 41, 65 and 95% of hesperidin originally present in *C. intermedia*, *C. subternata* and *C. sessiliflora*, respectively, remained after fermentation.

Table 1. Total soluble solids and phenolic contents[a] of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species.

Species	Soluble solids[b]		Total polyphenols[c]		Flavanols[d]		Flavonols + Flavones[e]	
	Unfermented	*Fermented*	*Unfermented*	*Fermented*	*Unfermented*	*Fermented*	*Unfermented*	*Fermented*
C. intermedia[f]	27.24c ± 1.35	16.96e ± 0.76	30.28b ± 0.48 (8.25)	16.26d ± 1.39 (2.76)	**2.34a ± 0.14 (0.64)**	**0.61d ± 0.16 (0.10)**	3.27ef ± 0.39 (0.89)	2.62g ± 0.37 (0.44)
C. subternata[f]	**37.60a ± 4.32**	22.19d ± 1.49	**32.41a ± 0.45 (12.19)**	17.16d ± 1.59 (3.81)	1.23b ± 0.17 (0.46)	0.18f ± 0.03 (0.04)	3.45e ± 0.49 (1.29)	2.85fg ± 0.16 (0.63)
C. sessiliflora[g]	33.14b ± 1.54	21.94d ± 1.16	29.52b ± 1.27 (9.78)	16.76d ± 1.59 (3.68)	0.77c ± 0.05 (0.26)	0.14f ± 0.03 (0.03)	6.56c ± 0.34 (2.17)	4.40d ± 0.38 (0.97)
C. genistoides[g]	**39.01a ± 3.6**	**34.91b ± 2.09**	28.96b ± 1.18 (11.71)	**21.89c ± 0.89 (7.64)**	0.87c ± 0.09 (0.35)	0.41e ± 0.03 (0.05)	**8.50a ± 0.58 (3.44)**	**7.58b ± 0.46 (2.65)**

[a] Means within the same assay followed by the same letter are not significantly different ($P > 0.05$).
[b] Expressed as g soluble solids 100 g^{-1} plant material.
[c] Expressed as g gallic acid equivalents 100 g^{-1} DAE and g gallic acid equivalence 100 g^{-1} plant material in brackets as determined using Folin-Ciocalteu reagent.
[d] Expressed as g catechin equivalents 100 g^{-1} DAE and g catechin equivalents 100 g^{-1} plant material in brackets.
[e] Expressed as g quercetin equivalents 100 g^{-1} DAE and g quercetin equivalents 100 g^{-1} plant material in brackets.
[f] Each value represents the mean ± S.D. of 6 replicates with triplicate analysis.
[g] Each value represents the mean ± S.D. of 7 replicates with triplicate analysis.
Unfermented species with the highest mean.
■ Fermented species with the highest mean.

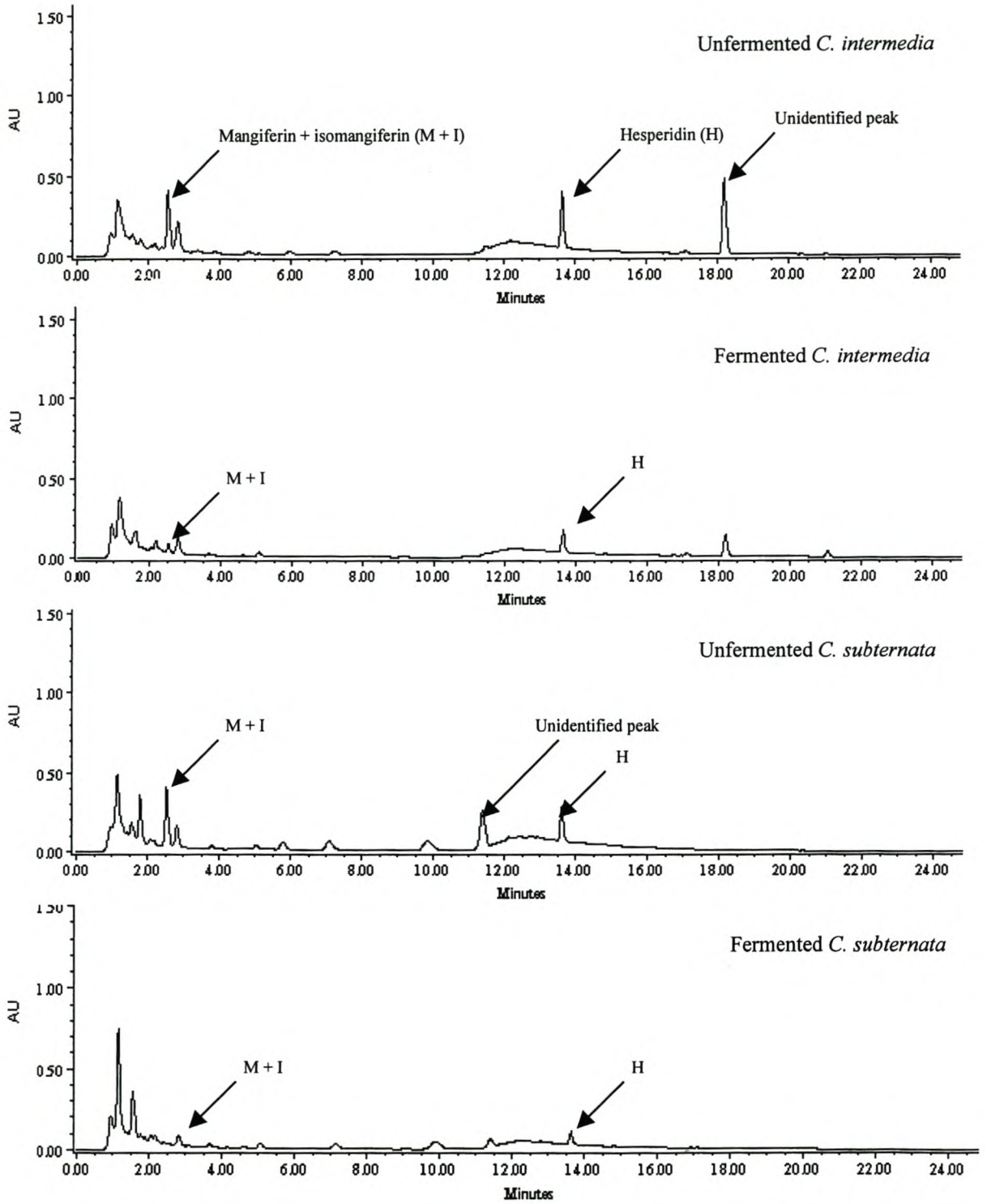


Figure 1. Typical chromatograms of the dry aqueous extract of unfermented and fermented *C. intermedia* and *C. subternata*. HPLC analysis was carried out on a Zorbax SB-C_{18} (3.5 µm particle size, 150 x 3 mm) column under gradient conditions; mobile phase, acetic acid:acetonitrile; flow rate, 1 mL min^{-1}; detection, 280 nm; column temperature, 30°C.

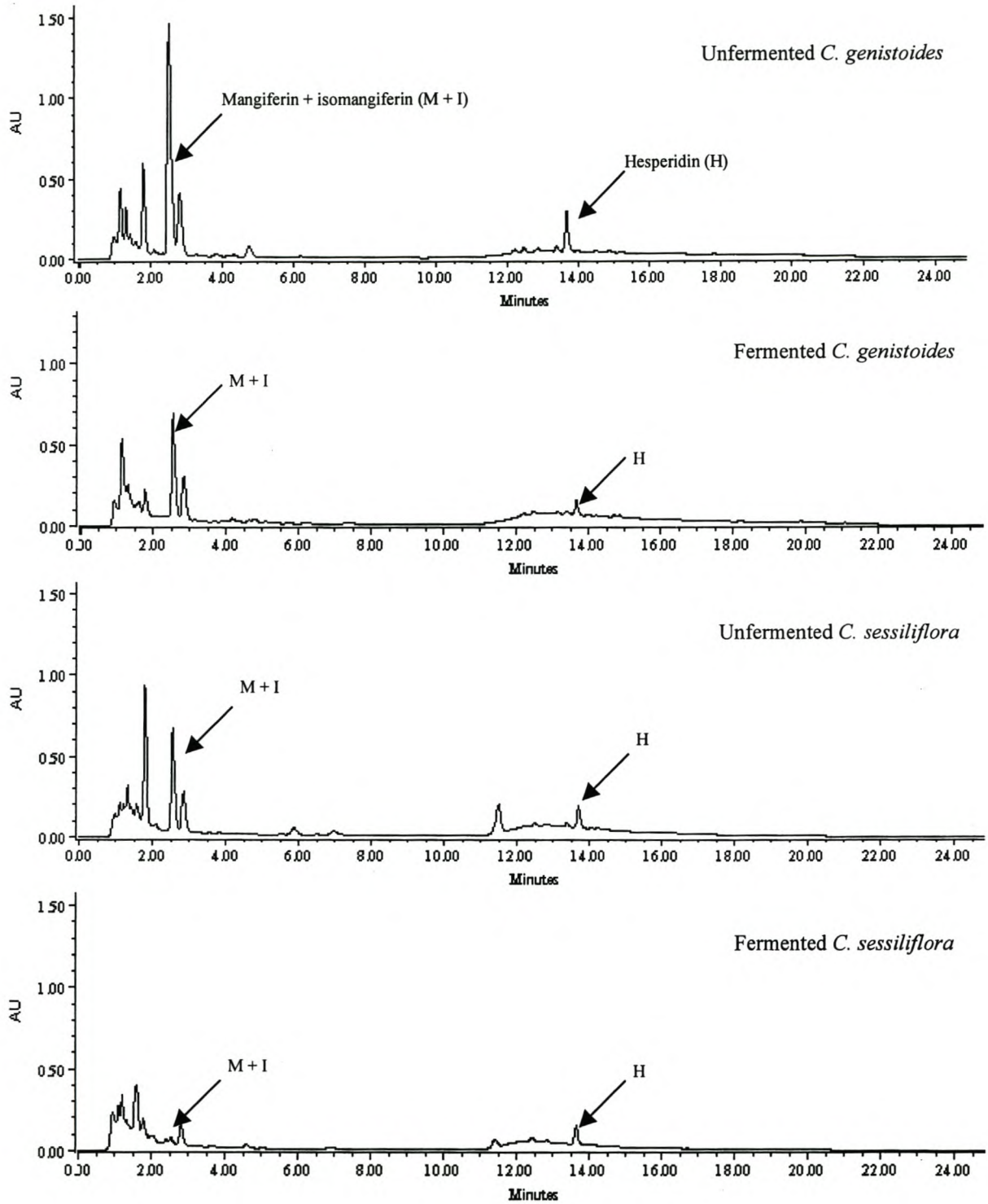


Figure 2. Typical chromatograms of the dry aqueous extract of unfermented and fermented *C. genistoides* and *C. sessiliflora*. HPLC analysis was carried out on a Zorbax SB-C_{18} (3.5 µm particle size, 150 x 3 mm) column under gradient conditions; mobile phase, acetic acid:acetonitrile; flow rate, 1 mL min^{-1}; detection, 280 nm; column temperature, 30°C.

Table 2. Quantification of the major phenolic compounds of the dry aqueous extracts (DAE) of fermented and unfermented *Cyclopia* species through HPLC analysis[a].

Species	Mangiferin + isomangiferin		Hesperidin	
	Unfermented	*Fermented*	*Unfermented*	*Fermented*
C. intermedia[b]	2.86c ± 0.69 (0.78)	0.33e ± 0.13 (0.01)	1.3a ± 0.11 (0.35)	0.53c ± 0.07 (0.09)
C. subternata[b]	1.69d ± 0.43 (0.64)	0.19e ± 0.12 (0.04)	0.48cd ± 0.2 (0.18)	0.31e ± 0.09 (0.07)
C. sessiliflora[c]	5.38b ± 0.47 (1.78)	0.31e ± 0.09 (0.07)	0.58c ± 0.05 (0.19)	0.55c ± 0.05 (0.12)
C. genistoides[c]	11.69a ± 0.88 (4.56)	4.69b ± 1.46 (1.63)	1.05b ± 0.12 (0.41)	0.38de ± 0.02 (0.13)

[a] Mangiferin + isomangiferin were quantified as mangiferin due to their co-elution. Analysis was done in duplicate using reversed-phase C_{18} and an acetic acid:acetonitrile elution gradient.

[b] Each value represents the mean (g 100 g^{-1} DAE) ± S.D. of 6 replicates (harvested bundles of plant material) with duplicate analysis. Means for unfermented and fermented DAE followed by the same letter are not significantly different ($P > 0.05$). Means in brackets are g 100 g^{-1} plant material.

[c] Each value represents the mean (g 100 g^{-1} DAE) ± S.D. of 7 replicates (harvested bundles of plant material) with duplicate analysis. Means for unfermented and fermented teas followed by the same letter are not significantly different ($P > 0.05$). Means in brackets are g 100 g^{-1} plant material.

The ratio of mangiferin to gallic acid and hesperidin to gallic acid required to give the same colour reaction with the Folin-Ciocalteu reagent (Figure 3) was 0.47:1 and 0.98:1, respectively. Figures 4 and 5 show the contribution of mangiferin + isomangiferin (assuming isomangiferin has a similar reactivity towards the Folin-Ciocalteu reagent as mangiferin) and hesperidin to the total polyphenol content of the DAE. Mangiferin + isomangiferin, present in the highest quantities in unfermented *C. genistoides*, contributed 86% of the total polyphenol content of the DAE. For hesperidin, present in the highest quantity in unfermented *C. intermedia*, the apparent contribution to the total polyphenols was only 4.3%. After fermentation the apparent contribution of mangiferin + isomangiferin of *C. genistoides* was 46%, compared to 2.3-4.3% of the other species.

Radical scavenging and ferric reducing ability as a measure of total antioxidant activity

Antioxidant activity expressed as the radical scavenging ability in the $ABTS^{\bullet+}$ assay and ferric reducing ability in the FRAP assay decreased significantly ($P < 0.05$) for all species after fermentation (Table 3). According to the $ABTS^{\bullet+}$ assay no significant difference existed between the DAE of unfermented *C. intermedia* and *C. subternata* and *C. subternata* with *C. sessiliflora* and *C. genistoides*. Fermented *C. genistoides* had a significantly ($P < 0.05$) higher $ABTS^{\bullet+}$ scavenging activity than the other species. The distinction between the species was slightly different with the FRAP assay. In this case unfermented *C. genistoides* was significantly less active that the other species. However similar to the $ABTS^{\bullet+}$ assay *C. genistoides* was the most active of the fermented species, with no significant ($P > 0.05$) difference between the other species.

The antioxidant potency of the total polyphenols, obtained by expressing the antioxidant activity on an equal gallic acid equivalents basis as opposed to an equal DAE mass basis, decreased significantly with fermentation for all species, irrespective of the assay used. The polyphenols of unfermented *C. intermedia, C. sessiliflora* and *C. genistoides* had the higher potency in the $ABTS^{\bullet+}$ assay, whereas only the first two species were the most potent in the FRAP assay. Of the fermented teas *C. genistoides* had the most potent polyphenols in the $ABTS^{\bullet+}$ assay. The polyphenols of fermented *C. sessiliflora* and *C. subternata* were the least potent. The polyphenol potency of the fermented species were shown not to differ significantly in the FRAP assay.

The apparent contribution of mangiferin + isomangiferin to the $ABTS^{\bullet+}$ scavenging ability of the DAE was calculated relative to the activity of Trolox (Figure 6). The

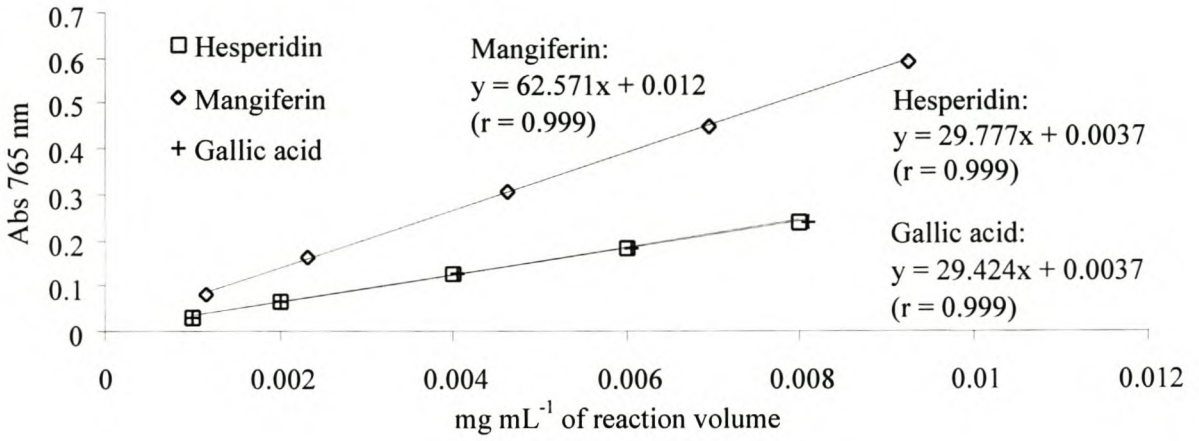


Figure 3. Relative reactivities of mangiferin, hesperidin and gallic acid toward the Folin-Ciocalteu reagent.

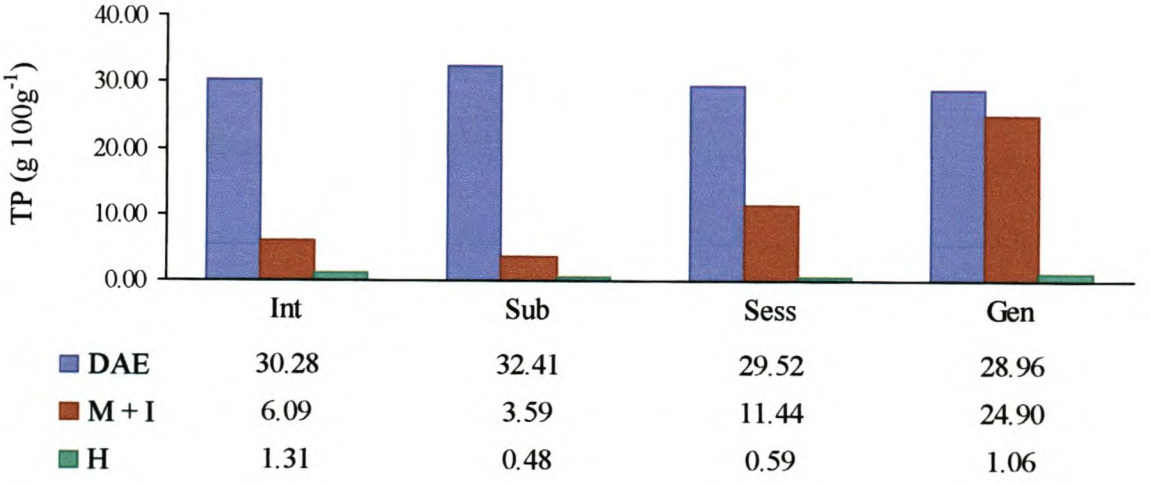


Figure 4. Apparent contribution of mangiferin + isomangiferin (M + I) and hesperidin (H) to total polyphenol content (TP) (g gallic acid equivalents 100 g^{-1} DAE) of the dry aqueous extracts (DAE) of unfermented *Cyclopia* species [*C. intermedia* (Int), *C. subternata* (Sub), *C. sessiliflora* (Sess) and *C. genistoides* (Gen)]. The relationship between the reactivity of mangiferin and hesperidin towards the Folin-Ciocalteu reagent relative to that of gallic acid was 0.47 and 0.99, respectively (as g required to give an equal response as 1 g gallic acid).

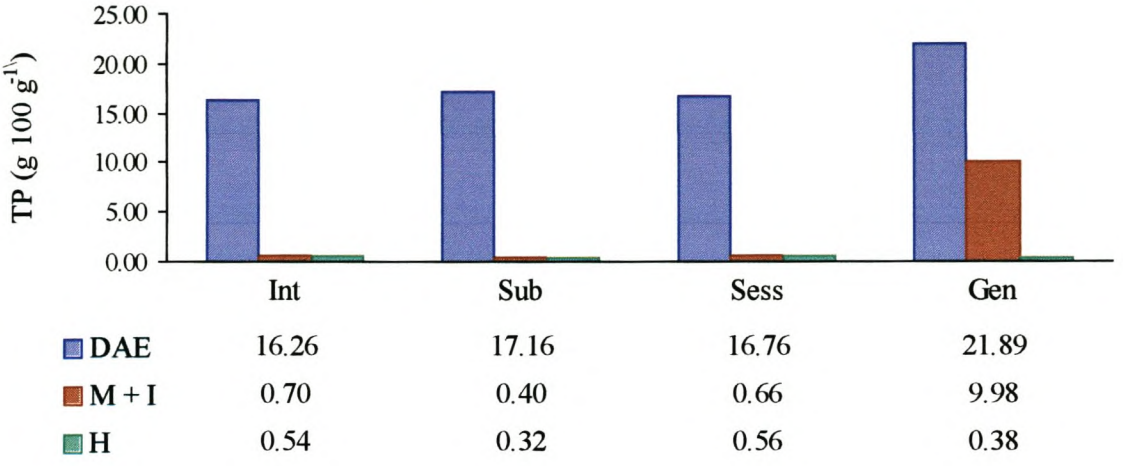


Figure 5. Apparent contribution of mangiferin + isomangiferin (M + I) and hesperidin (H) to total polyphenol content (TP) (g gallic acid equivalents 100 g^{-1} DAE) of the dry aqueous extracts (DAE) of fermented *Cyclopia* species [*C. intermedia* (Int), *C. subternata* (Sub), *C. sessiliflora* (Sess) and *C. genistoides* (Gen)]. The relationship between the reactivity of mangiferin and hesperidin towards the Folin-Ciocalteu reagent relative to that of gallic acid was 0.47 and 0.99, respectively (as g required to give an equal response to 1 g gallic acid)

Table 3. Radical scavenging and ferric reducing abilities[a] of the dried aqueous extract (DAE) of unfermented and fermented *Cyclopia* species as a measure of total antioxidant activity in the $ABTS^{\bullet+}$ decolourisation and FRAP assays, respectively.

Species	TAA[b]		TAA/TP[c]		FRAP[d]		FRAP/TP[e]	
	Unfermented	*Fermented*	*Unfermented*	*Fermented*	*Unfermented*	*Fermented*	*Unfermented*	*Fermented*
C. intermedia[f]	**2106.4a ± 73.9**	957.9d ± 77.5	**6956.4a ± 202.1**	5894.6d ± 73.7	**3399.0a ± 129.8**	1498.7d ± 129.9	**11224.1a ± 325.1**	**9219.7c ± 123.5**
C. subternata[f]	**2032.8ab ± 64.4**	918.7d ± 78.5	6272.6c ± 165.9	5358.2e ± 145.2	**3316.0a ± 77.1**	1517.2d ± 172.2	10232.5b ± 178.5	**8828.8c ± 309.9**
C. sessiliflora[g]	1983.4b ± 104.5	936.1d ± 81.8	6723.8ab ± 386.8	5593.3e ± 277.6	**3278.1a ± 170.6**	1529.0d ± 164.8	**11104.8a ± 433.9**	**9123.4c ± 466.3**
C. genistoides[g]	2014.3b ± 59.7	**1443.2c ± 66.2**	**6960.2a ± 208.9**	**6600.3b ± 384.4**	2953.9b ± 76.5	**1953.3c ± 140.9**	10213.9b ± 480.3	**8929.9c ± 674.1**

[a] Means within the same assay followed by the same letter are not significantly different ($P > 0.05$).
[b] Total antioxidant activity expressed as µmol Trolox equivalents g^{-1} DAE.
[c] Polyphenol potency expressed as µmol Trolox equivalents g^{-1} gallic acid equivalents.
[d] Ferric reducing antioxidant power (FRAP) expressed as µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} DAE.
[e] Polyphenol potency expressed as µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} gallic acid equivalents.
[f] Each value represents the mean ± S.D. of 6 replicates with triplicate analysis.
[g] Each value represents the mean ± S.D. of 7 replicates with triplicate analysis.
bold Unfermented species with the highest mean.
■ Fermented species with the highest mean.

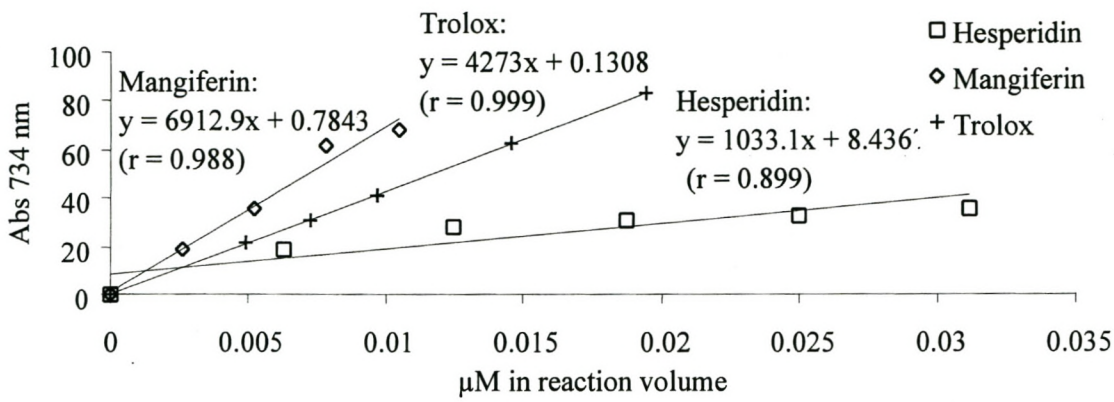


Figure 6. Relative activities of mangiferin, hesperidin and Trolox in scavenging the ABTS radical cation in the $ABTS^{\bullet+}$ decolourisation assay.

concentrations (μmol) required to give the same activity as 1 μmol Trolox were found as 0.618 for mangiferin + isomangiferin and 4.128 for hesperidin. Mangiferin + isomangiferin had the highest apparent contribution to the $ABTS^{\bullet+}$ scavenging activity of *C. genistoides* (Figures 7 and 8) and with fermentation the apparent contribution decreased from 22 to 12%. Hesperidin contributed 0.3% to the $ABTS^{\bullet+}$ scavenging activity of both the unfermented and fermented *Cyclopia* species.

The relative antioxidant activity as ferric reducing power of mangiferin and hesperidin to iron(II)sulphate heptahydrate as μmol required to give the same activity as 1 μmol $FeSO_4 \bullet 7H_2O$ were found as 0.225 for mangiferin + isomangiferin and 0.448 for hesperidin (Figure 9). The apparent contribution of these compounds to the ferric reducing ability of the DAE of the unfermented and fermented *Cyclopia* species was more substantial for mangiferin + isomangiferin than for hesperidin (Figure 10 and 11). The apparent contribution of mangiferin + isomangiferin to the ferric reducing power of unfermented *C. genistoides* and *C. sessiliflora* was *c.* 42 and 18%, respectively, while even lower values were obtained for *C. intermedia* (*c.* 9%) and *C. sessiliflora* (*c.* 5%). Hesperidin made only a small contribution (*c.* 0.5-1.5%) to the ferric reducing ability of the DAE of the unfermented species. The apparent contribution of mangiferin + isomangiferin to the ferric reducing ability of fermented *C. genistoides* was *c.* 25%, but for the other species the apparent contribution was less than 2.5%. Hesperidin contributed less than 1.4% of the ferric reducing activity of the DAE of the fermented *Cyclopia* species.

Inhibition of microsomal lipid peroxidation

Fermentation affected the ability of the DAE to inhibit lipid peroxidation significantly ($P < 0.05$) (Table 4). Unfermented species had a higher protective effect against lipid peroxidation than their fermented counterparts, except in the case of *C. genistoides* where fermentation had no significant effect. Of the unfermented species *C. sessiliflora* gave the highest inhibition of lipid peroxidation followed by *C. subternata* and *C. intermedia*, which did not differ significantly, and *C. genistoides* that was the least protective. However, with fermentation *C. genistoides* retained its ability (*c.* 32%) to inhibit lipid peroxidation, while that of *C. intermedia*, *C. subternata*, and *C. sessiliflora* was reduced to the same level at *c.* 24%. Expressing the ability to inhibit lipid peroxidation on an equal gallic acid equivalents basis as opposed to DAE mass basis, shows that the potency of the total polyphenols increases with fermentation for all species, except for *C. sessiliflora*, which remained the same. However, the polyphenol potency of the fermented species did not differ significantly.

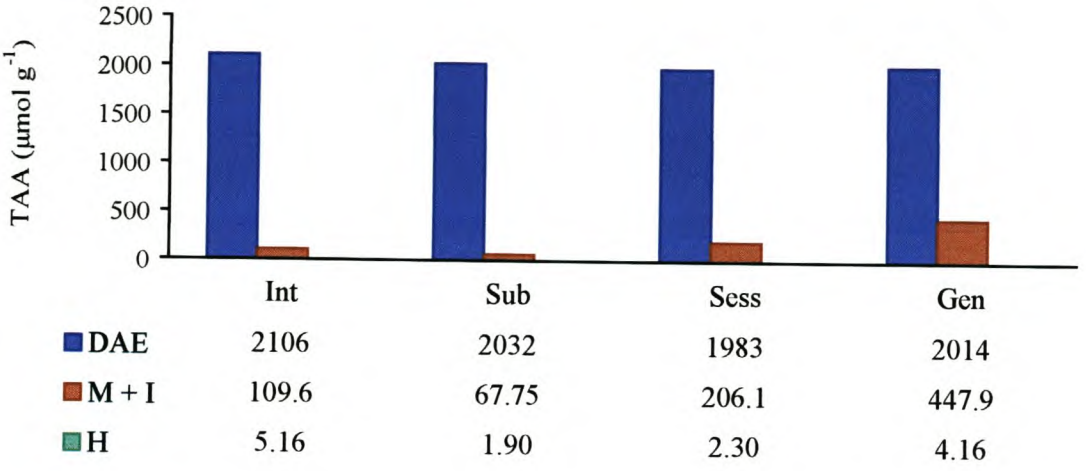


Figure 7. Apparent contribution of mangiferin + isomangiferin (M + I) and hesperidin (H) to the $ABTS^{\bullet+}$ scavenging activity (TAA) as µmol Trolox equivalents g^{-1} dry aqueous extracts (DAE) of unfermented *Cyclopia* species [*C. intermedia* (Int), *C. subternata* (Sub), *C. sessiliflora* (Sess) and *C. genistoides* (Gen)]. The relative concentration required to obtain the same activity for mangiferin, hesperidin and Trolox was 0.618:4.128:1, respectively.

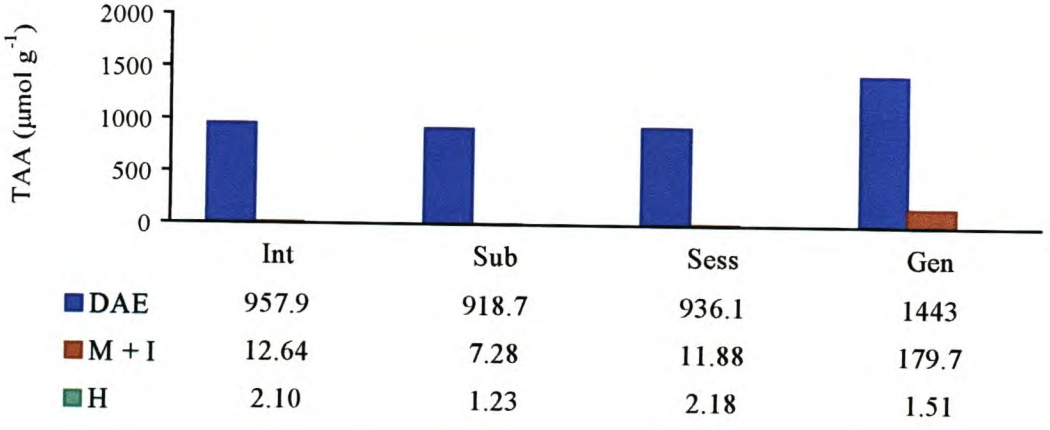


Figure 8. Apparent contribution of mangiferin + isomangiferin (M + I) and hesperidin (H) to the $ABTS^{\bullet+}$ radical scavenging activity (TAA) as µmol Trolox equivalents g^{-1} dry aqueous extracts (DAE) of fermented *Cyclopia* species [*C. intermedia* (Int), *C. subternata* (Sub), *C. sessiliflora* (Sess) and *C. genistoides* (Gen)]. The relative concentration required to obtain the same activity for mangiferin, hesperidin and Trolox was 0.618:4.128:1, respectively.

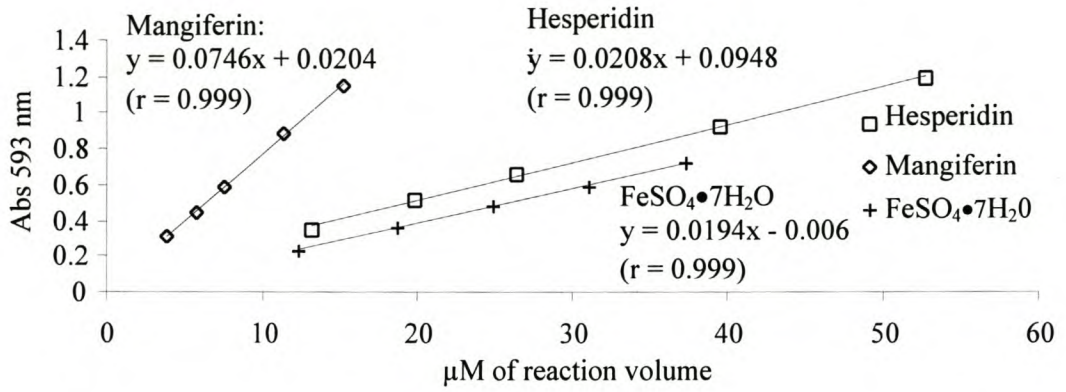


Figure 9. Relative activities of mangiferin, hesperidin and iron(II)sulphate heptahydrate for the reduction of the Fe(III)-2,4,5-tri(2-pyridyl)-S-triazine complex in the ferric reducing antioxidant power assay.

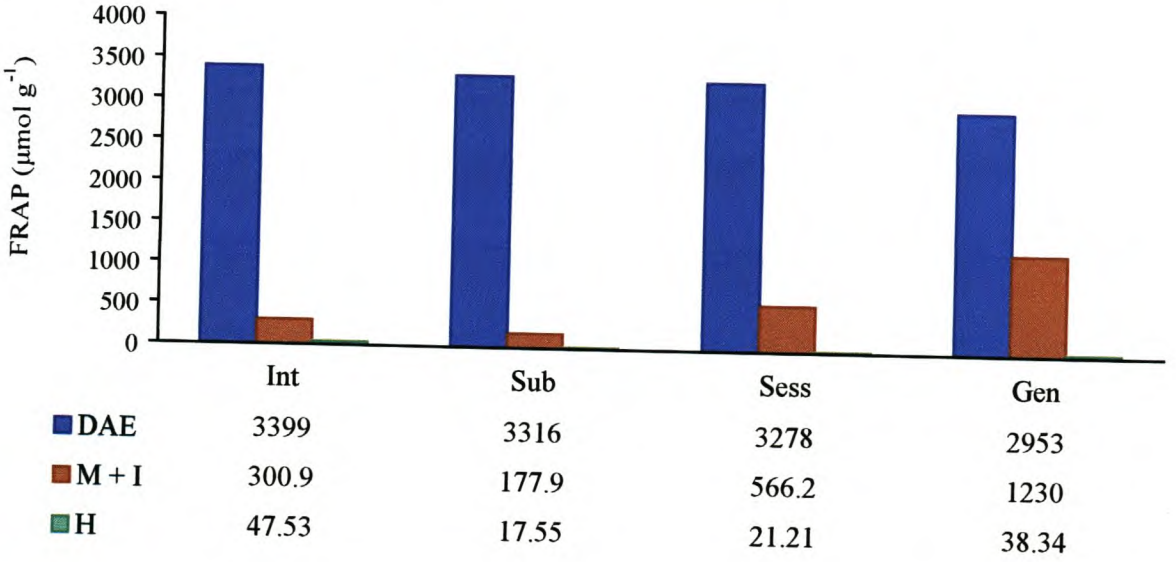
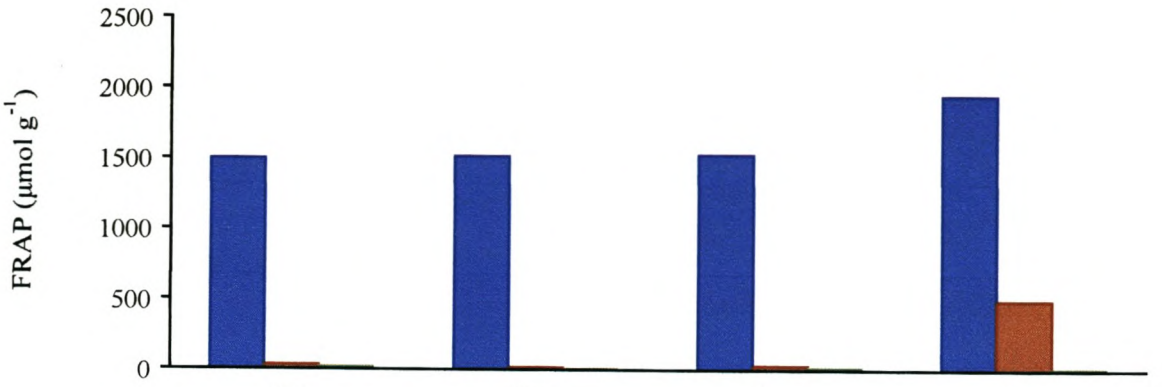


Figure 10. Apparent contribution of mangiferin + isomangiferin (M + I) and hesperidin (H) to the ferric reducing antioxidant power (FRAP) as µmol $FeSO_4 \bullet H_2O$ equivalents g^{-1} dry aqueous extracts (DAE) of unfermented *Cyclopia* species [*C. intermedia* (Int), *C. subternata* (Sub), *C. sessiliflora* (Sess) and *C. genistoides* (Gen)]. The relative concentration required to obtain the same activity for mangiferin, hesperidin and $FeSO_4 \bullet 7H_2O$ was 0.225:0.448:1, respectively.



	Int	Sub	Sess	Gen
DAE	1498	1517	1529	1953
M + I	31.26	19.99	32.63	493.6
H	19.37	11.33	20.11	13.89

Figure 11. Apparent contribution of mangiferin + isomangiferin (M + I) and hesperidin (H) to the ferric reducing antioxidant power (FRAP) as µmol $FeSO_4 \bullet H_2O$ equivalents g^{-1} dry aqueous extracts (DAE) of fermented *Cyclopia* species [*C. intermedia* (Int), *C. subternata* (Sub), *C. sessiliflora* (Sess) and *C. genistoides* (Gen)]. The relative concentration required to obtain the same activity for mangiferin, hesperidin and $FeSO_4 \bullet 7H_20$ was 0.225:0.448:1, respectively.

Table 4. Inhibition of Fe^{2+} induced rat liver microsomal lipid peroxidation of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species.

Species	TBARS[ab]		TBARS/TP[bc]	
	Unfermented	*Fermented*	*Unfermented*	*Fermented*
C. intermedia[d]	36.34b ± 1.81	23.63d ± 3.29	39.91b ± 1.69	48.29a ± 5.46
C. subternata[d]	36.21b ± 2.40	23.93d ± 4.92	37.15b ± 2.61	**46.48a ± 9.22**
C. sessiliflora[e]	**41.91a ± 3.76**	23.77d ± 2.37	**47.36a ± 6.01**	**47.24a ± 3.94**
C. genistoides[e]	30.89c ± 3.85	**32.28c ± 3.23**	35.43b ± 3.81	**48.95a ± 3.58**

[a] Percentage inhibition of lipid peroxidation measured as thiobarbituric reactive substances (TBARS) on an equal DAE mass basis.
[b] Means for unfermented and fermented species followed by the same letter are not significantly different ($P > 0.05$).
[c] Percentage inhibition of lipid peroxidation measured as thiobarbituric reactive substances (TBARS) on an equal total polyphenol content.
[d] Each value represents the mean ± S.D. of 6 replicates with triplicate analysis.
[e] Each value represents the mean ± S.D. of 7 replicates with triplicate analysis.
Unfermented species with the highest mean.
Fermented species with the highest mean.

Mangiferin and hesperidin were poor inhibitors of microsomal lipid peroxidation. Concentrations of mangiferin and hesperidin far greater than those of the DAE were required to inhibit microsomal lipid peroxidation effectively (Figure 12). Even at concentration as high as 0.2 mg mL^{-1} (in the reaction mixture), which was much higher than the concentration of mangiferin and hesperidin present in the DAE, mangiferin inhibited lipid peroxidation by only 48% and hesperidin by only 15%.

Antimutagenic activity (*S. typhimurium* antimutagenicity assay)

The antimutagenic activity of the *Cyclopia* species varied to a large extent. At 2 mg DAE per plate unfermented *C. intermedia* and *C. sessiliflora* gave the best protection against mutagenesis. *Cyclopia genistoides* was promutagenic in the presence of metabolically activated 2-AAF, since 613.43 revertants formed compared to 393.98 of the positive control (Table 5). However, it is important to note that unfermented *C. genistoides* did not give a mutagenic response in the presence and absence of S9 against TA98 at concentrations of 1 and 2 mg $plate^{-1}$ (Table 6). Fermentation resulted in a decrease of the protective effect of *C. intermedia* and *C. sessiliflora*, but in the case of *C. subternata* their was no significant decrease in the protective effect with fermentation. Fermentation decreased the number of revertants given by *C. genistoides* so that it was not promutagenic, but together with fermented *C. intermedia* it gave the highest number of revertants and therefore provided the least protection against 2-AAF induced mutagenesis (Table 5).

Comparison of the unfermented and fermented species at different concentrations (1 and 2 mg $plate^{-1}$ for unfermented and 2 and 5 mg $plate^{-1}$ for fermented) revealed a dose response effect against the metabolically activated mutagen 2-AAF (Figures 13 and 14). An increase from 1 to 2 mg DAE $plate^{-1}$ increased the percentage inhibition of unfermented *C. intermedia*, *C. subternata* and *C. sessiliflora,* while *C. genistoides* had an increased promutagenic response at the higher DAE concentration. In the case of the fermented species *c.* 5 mg DAE was needed to give *c.* 80% inhibition compared to *c.* 2 mg DAE for the unfermented species. Fermented *C. genistoides* was also not promutagenic at the level of 5 mg DAE per plate (Figure 14). Fermented *C. sessiliflora* had the highest antimutagenic activity at both DAE concentrations (75.43 and 91% inhibition for 2 and 5 mg DAE $plate^{-1}$, respectively). The relative difference in activity observed at the different concentrations differed amongst species. Fermented *C. genistoides* and *C. intermedia* experienced the greatest change in activity with an increase in the concentration. The ability to inhibit mutagenesis increased from *c.* 51 to 85% for *C.*

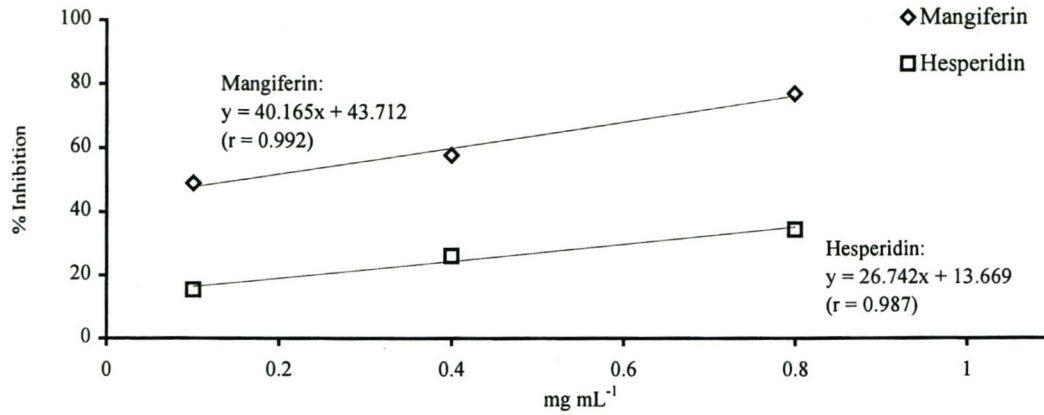


Figure 12. Ability of mangiferin and hesperidin to inhibit Fe^{2+} induced lipid peroxidation using rat liver microsomes as a model membrane.

Table 5. Protective effect of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species on metabolically activated 2-acetylaminoflourene (2-AAF) induced mutagenesis in TA98 in the *S. typhimurium* antimutagenicity assay.

Species	**Revertants**[ab]	
	Unfermented	*Fermented*
Negative Control	31.76e ± 2.1	
Positive Control	393.89b ± 31.9	
C. intermedia[c]	**52.83e ± 7.9**	188.50c ± 20.2
C. subternata[c]	91.67d ± 24.2	**119.17d ± 22.3**
C. sessiliflora[d]	**53.86e ± 19.6**	**99.14d ± 13.7**
C. genistoides[d]	613.43a ± 82.9	217.00c ± 27.0

[a] The mean number of 2-AAF induced revertants plate^{-1} was 393.89 ± 31.9 and the frequency of spontaneous reversion was 31.76 ± 2.1 revertants plate^{-1}. Concentration of mutagen and DAE were 5 µg plate^{-1} and 2 mg plate^{-1}, respectively.

[b] Means for both unfermented and fermented DAE followed by the same letter are not significantly different ($P > 0.05$).

[c] Each value represents the mean ± S.D. of 6 replicates (harvested bundles of plant material) with 5 assay repeats.

[d] Each value represents the mean ± S.D. of 7 replicates (harvested bundles of plant material) with 5 assay repeats.

Unfermented species with the highest inhibition.

■ Fermented species with the highest inhibition.

Table 6. Effect of dry aqueous extract (DAE) of unfermented *C. genistoides* on the number of revertants in TA98 with and without metabolic activation in the *Salmonella typhimurium* antimutagenicity assay.

Species	**Revertants**[a]			
	1 mg DAE		*2 mg DAE*	
	+ S9	*- S9*	*+ S9*	*- S9*
C. genistoides[b]	39.64 ± 4.22	34.33 ± 2.25	40.08 ± 4.54	31.8 ± 3.03
Negative Control[c]	31.11 ± 6.48			
Positive Control[d]	433.0 ± 43.26			

[a] Number of revertants at 1 and 2 mg DAE plate^{-1} with and without the addition of S9 using tester strain TA98.

[b] Each value represents the mean ± S.D. of 7 replicates (harvested bundles of plant material) with 5 assay repeats.

[c] Number of spontaneous revertants ± S.D in TA98.

[d] Number of revertants ± S.D. from mutagenesis induced by 5 µg plate^{-1} metabolically activated 2-acetylaminofluorene in TA98.

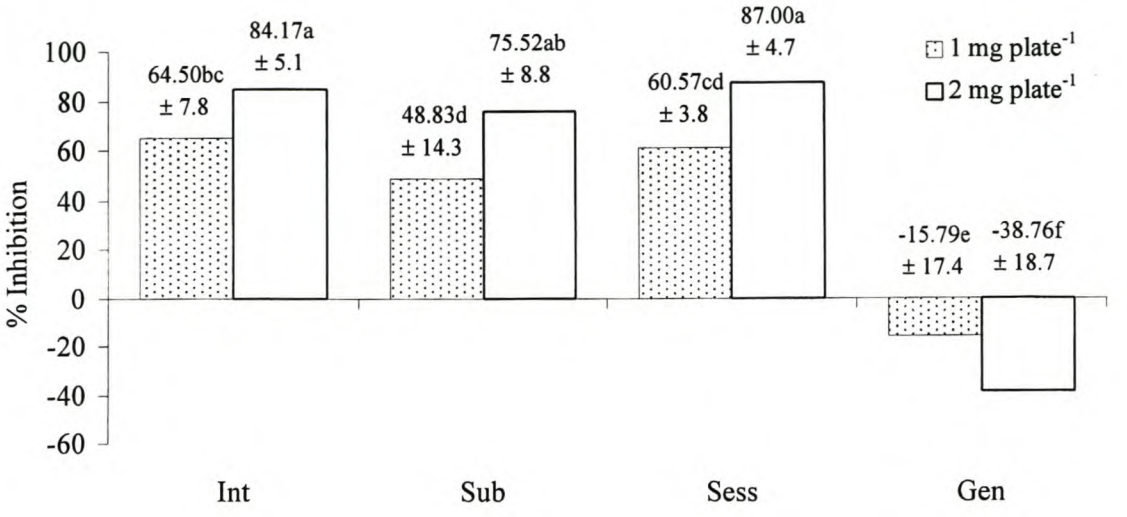


Figure 13. Antimutagenic activity of dry aqueous extract (DAE) of unfermented *Cyclopia* species against 2-AAF in the *S. typhimurium* antimutagenicity assay. Percentage inhibition at 1 and 2 mg DAE plate^{-1} are the mean ± S.D. of 6 [*C. intermedia* (Int) and *C. subternata* (Sub)] and 7 [*C. sessiliflora* (Sess) and *C. genistoides* (Gen)] replicates each done with 5 repeats in the analysis. Means within both concentrations with the same letter are not significantly different ($P > 0.05$).

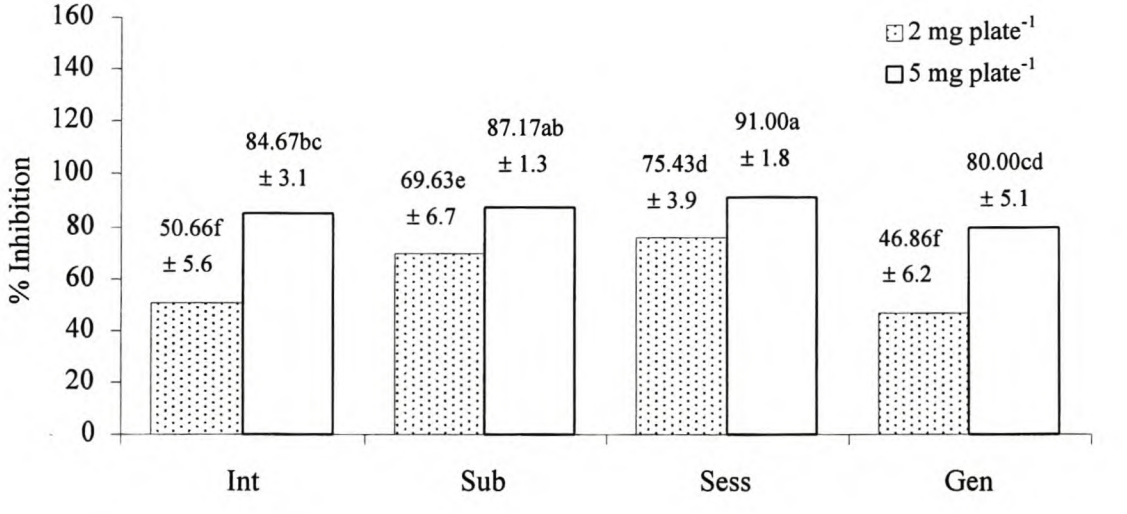


Figure 14. Antimutagenic activity of dry aqueous extract (DAE) of fermented *Cyclopia* species against 2-AAF in the *S. typhimurium* antimutagenicity assay. Percentage inhibition at 2 and 5 mg DAE plate^{-1} are the mean ± S.D. of 6 [*C. intermedia* (Int) and *C. dubternata* (Sub)] and 7 [*C. sessiliflora* (Sess) and *C. genistoides* (Gen)] replicates each done with 5 repeats in the analysis. Means within both concentrations with the same letter are not significantly different ($P > 0.05$)

intermedia and *c.* 47 to 80% for *C. genistoides* with an increase of DAE from 2 to 5 mg plate^{-1}. Although the increase for *C. subternata* and for *C. sessiliflora* was approximately half that of *C. genistoides* and *C. intermedia*, they were significantly more effective at the lower concentration (Figure 14).

Mangiferin and hesperidin had very poor antimutagenic activity against metabolically activated 2-AAF in the *S. typhimurium* antimutagenicity assay (Figure 15). At a concentration of 0.25 mg plate^{-1} mangiferin inhibited the number of spontaneous revertants by 4%, while hesperidin was promutagenic in the presence of 2-AAF and increased the number of spontaneous revertants by 8.8%. Hesperidin was found not to be mutagenic in the presence and absence of S9 with spontaneous revertants of 31-37 (with S9) and 18-32 (without S9) at concentrations of 0.25, 0.5, 1, 2 and 4 mg plate^{-1} within the same range as that of the negative control 34.4 ± 5.03.

Correlation of antioxidant and antimutagenic activity and phenolic content

All data obtained from the polyphenol analysis and antioxidant and antimutagenic assays were correlated with one another to obtain the relevant relationships between phenolic content and antioxidant and antimutagenic activity (Table 7).

Total polyphenol content correlated well with the antioxidant activity measured in the ABTS$^{\bullet+}$ ($r = 0.98$; $P < 0.0001$) and FRAP ($r = 0.98$; $P < 0.0001$) assays (Table 7). There is a distinct grouping of the unfermented versus the fermented teas in relation to total polyphenol content and antioxidant activity in the ABTS$^{\bullet+}$ and FRAP assays (Figures 16 and 17). A slightly weaker correlation was found between the flavanol content (Figures 18 and 19) and both of these assays. The greater variation in flavanol content of unfermented versus fermented *Cyclopia* with antioxidant activity is apparent in Figures 18 and 19. Values for unfermented *C.* intermedia are grouped separately from the other species. Although highly significant ($P < 0.0001$), weaker correlations were obtained between lipid peroxidation and total polyphenol ($r = 0.74$) and flavanol content ($r = 0.53$) than for the ABTS$^{\bullet+}$ and FRAP assays (Table 7). The correlations of the ability to inhibit lipid peroxidation with total polyphenol and flavanol contents are depicted in Figures 20 and 21, respectively. Similar to the other two antioxidant activity assays a definite separation between the species according to percentage inhibition based on flavanol content was observed (Figure 21). Mangiferin + isomangiferin and hesperidin contents could be moderately correlated ($r < 0.63$; $P < 0.0002$) with antioxidant activity (ABTS$^{\bullet+}$ and FRAP assays). Only very weak correlations between these compounds

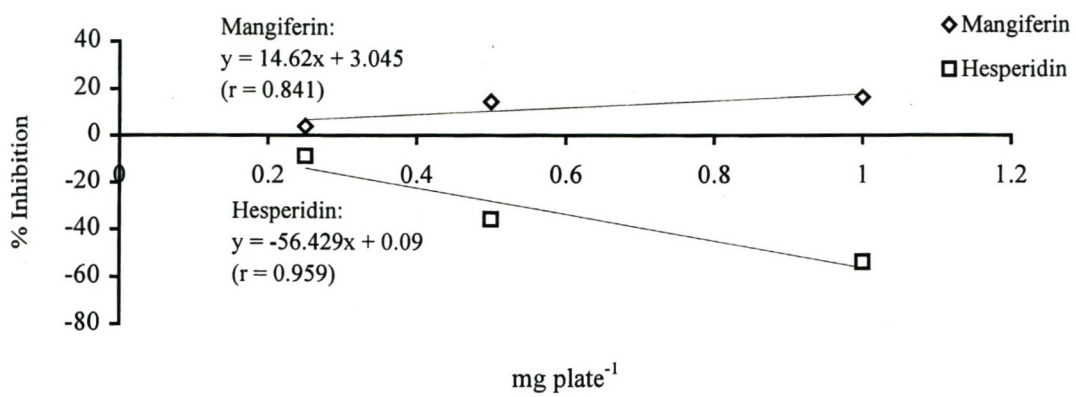


Figure 15. Antimutagenic activity of mangiferin and hesperidin over a concentration gradient against 2-AAF (5 μg plate^{-1}) using TA98 in the *S. typhimurium* antimutagenicity assay.

Table 7. Overall correlation coefficient between antioxidant activities[a], antimutagenic activity[b], total polyphenol content[c], flavanol content[d], flavonol + flavone content[e], mangiferin + isomangiferin content[f] and hesperidin content[g] of the dry aqueous extracts (DAE) of unfermented and fermented *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides*.

	ABTS	FRAP	LP	AMES
TP	0.98[h] (<0.0001)[i]	0.98 (<0.0001)	0.79 (<0.0001)	-0.082 (0.5630)
Flavanols	0.74 (<0.0001)	0.76 (<0.0001)	0.53 (<0.0001)	0.12 (0.3949)
Flavonols + flavones	0.40 (0.003)	0.27 (0.0556)	0.31 (0.0248)	-0.60 (<0.0001)
M + I	0.62 (<0.0001)	0.50 (0.0002)	0.36 (0.0085)	0.11[j] (P > 0.05)
H	0.59 (<0.0001)	0.57 (<0.0001)	0.28 (0.0434)	0.29 (0.0326)
ABTS		0.98 (<0.0001)	0.79 (<0.0001)	-0.16 (0.2668)
FRAP	0.98 (<0.0001)		0.80 (<0.0001)	-0.18 (0.8966)
LP	0.79 (<0.0001)	0.80 (<0.0001)		0.18 (0.2007)
AMES	-0.16 (0.2668)	-0.18 (0.8966)	0.18 (0.2007)	

[a] Radical scavenging ability and polyphenol potency (ABTS and ABTS/TP) in the $ABTS^{\bullet+}$ decolourisation assay, ferric reducing ability and polyphenol potency (FRAP and FRAP/TP) in the ferric reducing antioxidant power assay and ability to inhibit Fe^{2+}-induced rat liver microsomal lipid peroxidation model membranes and the polyphenol potency (LP and LP/TP).

[b] Antimutagenic activity (AMES) as percentage inhibition of 2 mg $plate^{-1}$ DAE towards 5 µg 2-acetylaminofluorene $plate^{-1}$ using TA98 in the *S. typhimurium* antimutagenicity assay.

[c] Total polyphenol content (TP) of the DAE using Folin-Ciocalteu reagent.

[d] Flavanol content of the DAE measured with dimethylaminocinnamaldehyde.

[e] Flavonol + flavone content of the DAE measured spectrophotometrically at 360 nm.

[f] Mangiferin + isomangiferin content (M + I) quantified using mangiferin as standard for reversed-phase (C_{18}) HPLC with an acetic acid:acetonitrile elution gradient.

[g] Hesperidin content (H) quantified using reversed-phase (C_{18}) HPLC with an acetic acid:acetonitrile elution gradient.

[h] Pearson's correlation coefficient.

[i] Probability.

[j] Correlation coefficient of antimutagenic activity versus mangiferin + isomangiferin content of all the species except unfermented *C. genistoides* that gave a promutagenic response.

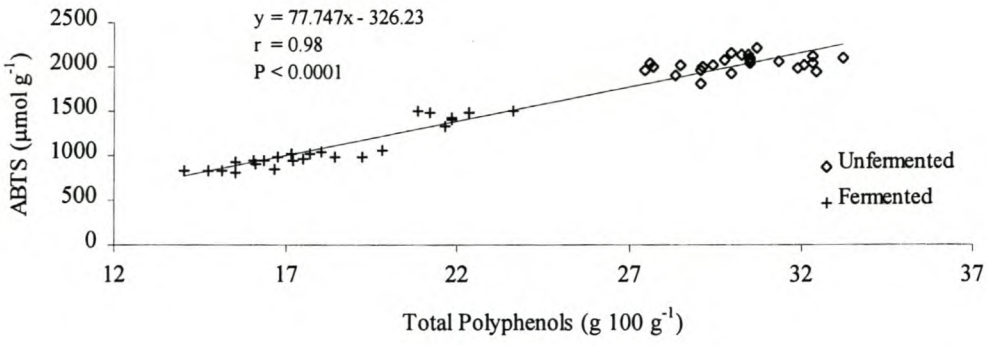


Figure 16. Correlation of radical scavenging ability (µmol Trolox equivalents g^{-1} DAE) with total polyphenol content (g gallic acid equivalents 100 g^{-1} DAE) of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species.

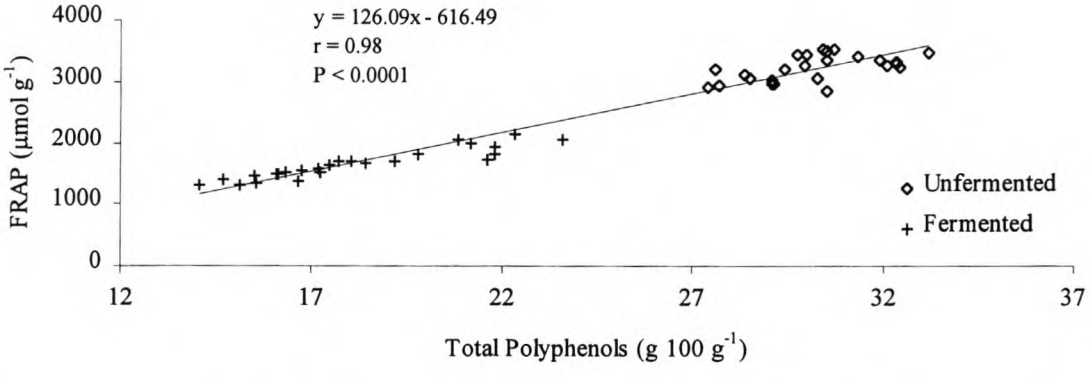


Figure 17. Correlation of ferric reducing antioxidant power (µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} DAE) with total polyphenol content (g gallic acid equivalents 100 g^{-1} DAE) of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species.

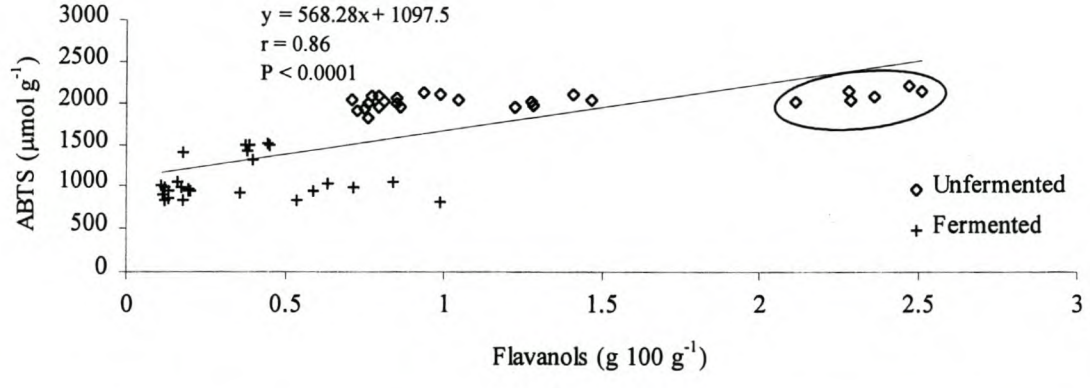


Figure 18. Correlation of radical scavenging ability (µmol Trolox equivalents g^{-1} DAE) with flavanol content (g gallic acid equivalents 100 g^{-1} DAE) of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species. Circled points are unfermented *C. intermedia.*

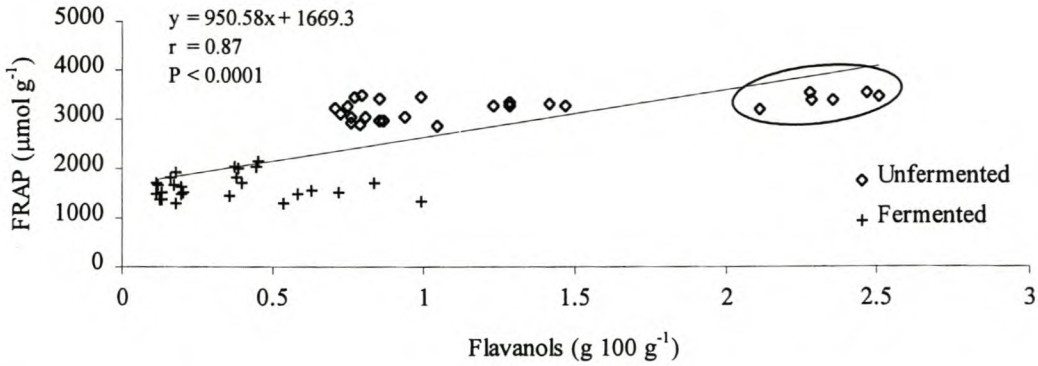


Figure 19. Correlation of ferric reducing antioxidant power (µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} DAE) with flavanol content (g gallic acid equivalents 100 g^{-1} DAE) of dried aqueous extract (DAE) of unfermented and fermented *Cyclopia* species. Circled points are unfermented *C. intermedia.*

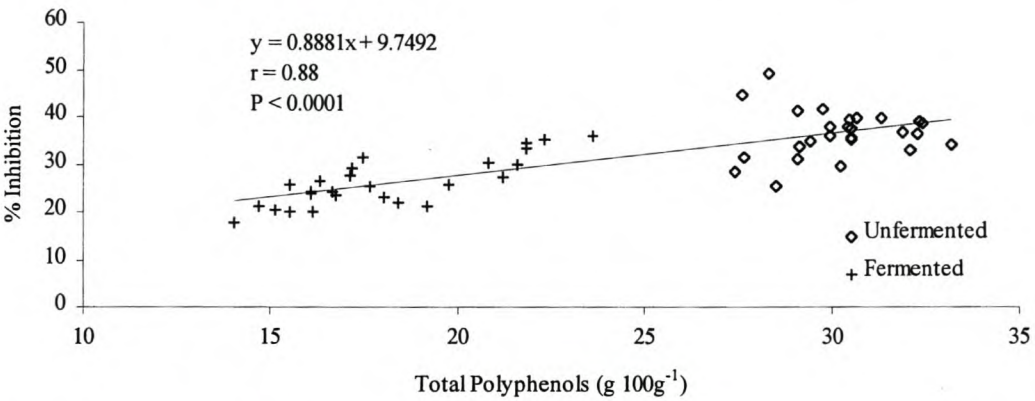


Figure 20. Correlation of ability to inhibit Fe^{2+} induced microsomal lipid peroxidation with total polyphenol content [g gallic acid equivalents 100 g^{-1} dry aqueous extract (DAE)] of DAE of unfermented and fermented *Cyclopia* species.

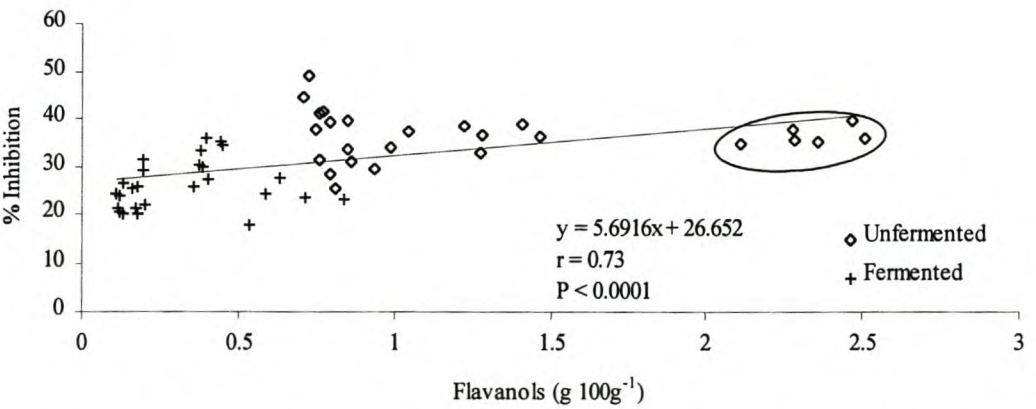


Figure 21. Correlation of the ability to inhibit Fe^{2+} induced microsomal lipid peroxidation with flavanol content [g gallic acid equivalents 100 g^{-1} dry aqueous extarct (DAE)] of DAE of unfermented and fermented *Cyclopia* species. Circled points are unfermented *C. intermedia.*

and inhibition of lipid peroxidation were observed (Table 7). A very good correlation coefficient ($r = 0.98$; $P < 0.0001$) was obtained for the antioxidant activity measured as ferric reducing ability versus the $ABTS^{\bullet+}$ scavenging ability (Figure 22). The $ABTS^{\bullet+}$ and FRAP assays had respective correlation coefficients of 0.79 and 0.8 with lipid peroxidation ($P < 0.0001$) (Figures 23 and 24). Antioxidant activity of the total polyphenols (polyphenol potency) correlated differently than the antioxidant activity of the soluble solids. Based on polyphenol potency the FRAP and $ABTS^{\bullet+}$ assays still showed a good correlation ($r = 0.74$; $P < 0.0001$) but the polyphenol potencies determined using these methods were poorly correlated with those determined with the microsomal lipid peroxidation assay [$r = -0.33$ ($P = 0.0167$) with the FRAP and $r = -0.23$ ($P = 0.0446$) with the $ABTS^{\bullet+}$ assays).

The antimutagenic activity significantly correlated with the flavonol + flavone ($r = 0.60$; $P < 0.0001$) content, but no correlation with total polyphenol or flavanol content was found (Table 7). Antimutagenic activity showed no correlation with the different antioxidant activity assays.

DISCUSSION

Natural and synthetic antioxidant compounds can exert a number of effects *in vivo* (German, 1998; Packer & Saliou, 1998; Waterhouse & Walzem, 1998). The feasibility of an extract or compound exerting antioxidant effects can be evaluated by *in vitro* tests that investigate how the putative antioxidant(s) can or cannot react with relevant free radicals (Frankel & Meyer, 2000). Although the $ABTS^{\bullet+}$ and FRAP assays are increasingly utilised to investigate antioxidant capacities of whole plant extracts (Ng *et al.*, 2000), more particularly vegetables [Velioglu *et al.*, 1998 ($ABTS^{\bullet+}$ assay)], teas [Rice-Evans *et al.*, 1997 ($ABTS^{\bullet+}$ assay); Benzie & Szeto, 1999 (FRAP assay)] and fruits [Deighton *et al.*, 2000 ($ABTS^{\bullet+}$ and FRAP assay); Netzel *et al.*, 2002 ($ABTS^{\bullet+}$ assay)], concerns continue to be expressed about the need to consider factors such as colloidal properties of substrates, conditions of the experimental methods, phase distribution of antioxidants and the physiological relevance of the assays (Aruoma & Cuppett, 1997; Frankel & Meyer, 2000; Schlesier *et al.*, 2002). In this study the *in vitro* antioxidant activity of aqueous extracts prepared from four different unfermented and fermented *Cyclopia* species was investigated for the first time using the $ABTS^{\bullet+}$, FRAP and Fe^{2+} induced microsomal lipid peroxidation assays. The study also provides the first

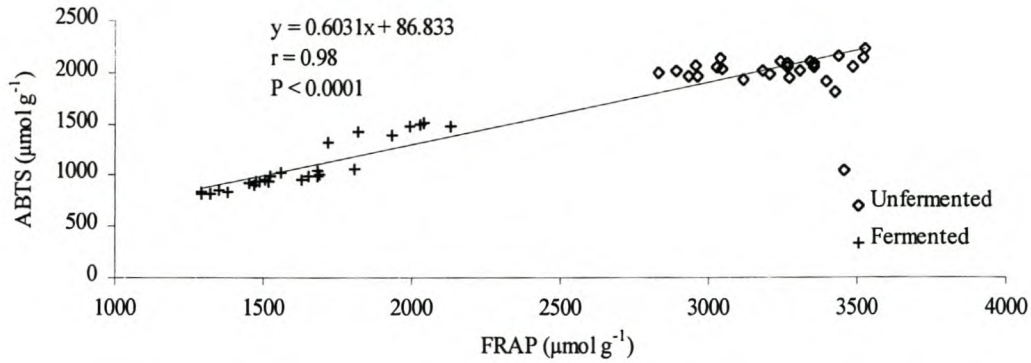


Figure 22. Correlation of radical scavenging ability (µmol Trolox equivalents g^{-1} DAE) with ferric reducing antioxidant power (µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} DAE) of dry aqueous extract (DAE) of unfermented and fermented *Cyclopia* species.

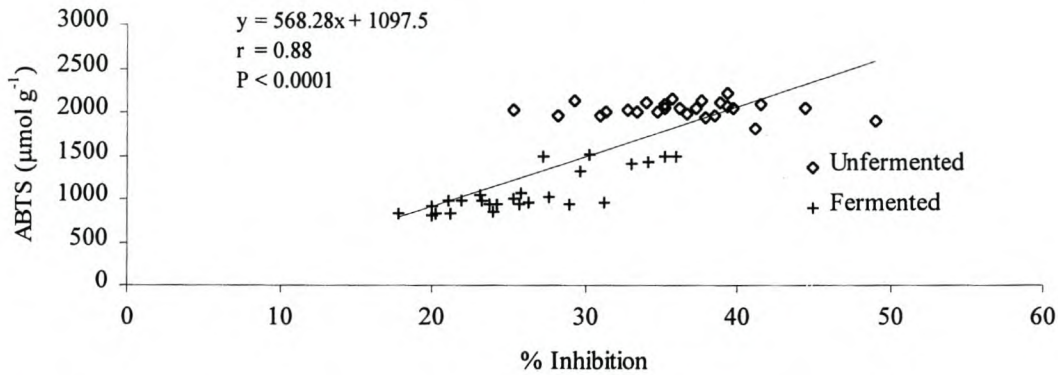


Figure 23. Correlation of radical scavenging ability [µmol Trolox equivalents g^{-1} dry aqueous extract (DAE)] with percentage inhibition of Fe^{2+} induced microsomal lipid peroxidation of DAE of unfermented and fermented *Cyclopia* species.

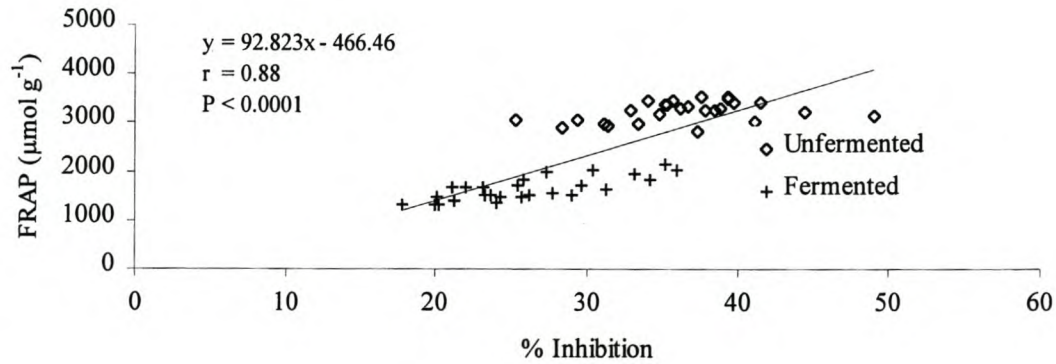


Figure 24. Correlation of and ferric reducing antioxidant power [µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} dry aqueous extract (DAE)] with percentage inhibition of Fe^{2+} induced microsomal lipid peroxidation of DAE of unfermented and fermented *Cyclopia* species.

comparative data on the antimutagenicity (*S. typhimurium* antimutagenicity assay) of the unfermented and fermented aqueous extracts of these commercially important *Cyclopia* species.

Their antioxidant and antimutagenic activities were correlated with their total polyphenol, flavanol, flavonol + flavone, mangiferin + isomangiferin and hesperidin contents. A significant correlation between the total polyphenols and antioxidant activity was shown for all three of the antioxidant assays used. Correlation coefficients for the $ABTS^{\bullet+}$ assay, FRAP assay and microsomal lipid peroxidation assay were 0.98, 0.98 and 0.79 at $P < 0.0001$, respectively. Similar correlation coefficients for the $ABTS^{\bullet+}$ ($r = 0.96$) and FRAP ($r = 0.82$) assays versus total polyphenols were obtained by Luximon-Ramma *et al.* (2002) for the fresh reproductive organs of *Cassia fistula*. Other studies also found correlation coefficients in the same order as these. The correlation of FRAP with the total polyphenol content of various juices (orange, grapefruit, apple, pineapple and vegetable) was $r = 0.98$ at $P < 0.0001$ (Gardner *et al.*, 2000) and $r = 0.86$ for fruit juice from *Rubus* species (Deighton *et al.*, 2000). The antioxidant capacity of the infusions prepared from 25 types of tea measured in the FRAP assay was also shown to strongly correlate with total polyphenol content ($r = 0.83$) (Benzie & Szeto, 1999). The $ABTS^{\bullet+}$ scavenging ability of fruit juice from *Rubus* species as measured in the $ABTS^{\bullet+}$ assay was moderately correlated with the total polyphenols ($r = 0.45$) (Deighton *et al.*, 2000). No previous studies on the correlation of total polyphenols with protective effect against Fe^{2+} induced microsomal lipid peroxidation could be found in the literature, but the correlation between antioxidant activity measured in the microsomal lipid peroxidation assay and total polyphenol content as found in this study is in agreement with similar correlations obtained for the protective effect of total polyphenols of grape wines ($r = 0.77$) (Frankel *et al.*, 1995) and grapes ($r = 0.62$) (Meyer *et al.*, 1997) against *in vitro* LDL oxidation. However, a very poor correlation (r ranged from 0.1-0.22) between total polyphenols and the inhibition of methyl linoleate peroxidation was found for berry and fruit wines and liquors (Heinonen *et al.*, 1998).

The correlation between inhibition of lipid peroxidation and total polyphenol content was not as strong as for the $ABTS^{\bullet+}$ scavenging and ferric reducing abilities ($r = 0.98$). However, a more complex reaction medium, i.e. consisting of both a hydrophobic and a hydrophilic phase, as well as their interface, is of importance in this regard. Furthermore, metal chelating ability will also affect the extent of oxidation of the microsomal lipid membrane. Flavonoid compounds with a catechol moiety in the B ring, the 3-hydroxyl and 4-

oxo groups in the heterocyclic ring and the 4-oxo and 5-hydroxyl groups between the heterocyclic and the A rings will chelate trace metals (Pietta, 2000). The different distribution patterns for the unfermented and fermented species were illustrated in the correlation graphs for $ABTS^{\bullet+}$ scavenging and ferric reducing abilities with inhibition of lipid peroxidation.

In the present study antimutagenicity against 2-AAF did not correlate with total polyphenol content of aqueous extracts. Yen & Chen (1995) also found no correlation between antimutagenic potential and total polyphenols, while the study by Marnewick *et al.* (2000) showed only a weak association between the total polyphenols and the antimutagenic effects when using the oxidative mutagens, H_2O_2 and cumolhydroperoxide (CHP) and the direct acting mutagen methyl methanesulfonate (MMS). No correlation was found between the antimutagenic activity measured against 2-AAF using tester strain TA98 and total polyphenol content of the aqueous extracts of unfermented and fermented *Cyclopia* species ($r = -0.082$; $P = 0.5630$). This indicates that the presence and number of hydroxyl groups are not the important factors, but that other properties such as the ability of the compounds to modulate the mutagenic response by modification of the permeability of bacterial membranes or by some extracellular physical, chemical or enzymatically catalysed interactions between flavonoids and mutagens as well as interactions between flavonoids and components of an exogenous mammalian metabolic system (i.e. S9) are crucial (Edenharder & Tang, 1997). A previous study (Yen & Chen, 1996) found that correlation coefficients between antimutagenic activity and the total polyphenols of *C. sinensis* tea extracts were not consistent (using a variety of mutagens and tester strains). Good correlations (r between 0.95-0.99; $P < 0.05$) were obtained for four of the five mutagens tested namely, benzo[*a*]pyrene (B[*a*]P), aflatoxin B_1 (AFB_1) and 2-amino-6-methyl-dipyrido(1,2-*a*:3',2'-*d*)imidazole (Glu-P-1) in TA98 and for B[*a*]P, AFB_1 and 2-amino-3-methylimidaza(4,5-*f*)quinoline (IQ) in TA100, but was determined by the tester strain used (Yen & Chen, 1996). A highly significant negative correlation, however, was observed between the antimutagenic activity and the flavonol + flavone content ($r = -0.60$; $P < 0.0001$).

Honeybush tea can be processed in two ways i.e. either the freshly harvested material is processed to inactivate the enzymatic oxidation enzymes for the production of green or unfermented teas (De Beer & Joubert., 2002) or the harvested material is "fermented" (Du Toit & Joubert, 1998) to favour chemical oxidative reactions for the production of traditional or fermented teas. A decrease in the yield of total soluble solids with fermentation, as shown in the present study, is attributed to decreases in its total polyphenol, flavanol and flavonol + flavone contents. Du Toit & Joubert (1998) and Hubbe (2000) also showed similar trends for

soluble solids and total polyphenol contents of *Cyclopia* species with fermentation. In the present study the total soluble solids of unfermented *C. genistoides* and *C. sessiliflora* had the lowest total polyphenol content, while Hubbe (2000) found the highest level in *C. sessiliflora*, followed by *C. genistoides*. These discrepancies in results could probably be attributed to different extraction conditions used, the leaf to stem ratio, the age of the plantation or the area grown. Up to date no studies have been undertaken to explain the compositional changes in the aqueous extract with fermentation on a fundamental basis. A possible explanation could be that heterogeneous polymers with reduced solubility are formed through the reaction of quinones with one another (Subramanian *et al* 1999). The quinones are formed when the polyphenols are oxidised by polyphenol oxidase released from the cell vacuole upon disruption of the intracellular compartments with shredding. With the application of heat during the fermentation process inactivation of the enzymes will terminate enzymatic oxidation reactions, but chemical oxidation will continue to occur and account for the majority of the changes during the high temperature-long fermentation period of honeybush tea (Du Toit & Joubert, 1998).

The extent to which fermentation affects the phenolic composition of the different *Cyclopia* species will depend on the individual compounds present in the plant material, as chemical structure will determine oxidation potentials (Prior & Cao, 1999). Investigations of the phenolic composition of fermented *C. intermedia* (Ferreira *et al.*, 1998; Kamara, 1999) and unfermented *C. subternata* (Brand, 2002) have shown them to be largely dissimilar. This was confirmed to a certain extent by HPLC analysis with the unidentified peaks at *c.* 11.4 and 18.2 min for unfermented *C. subternata* and *C. intermedia,* respectively. Epigallocatechin 3-*O*-gallate present in *C. subternata* is highly susceptible to oxidation (Roberts, 1957), while coumestans and isoflavones present in *C. intermedia* (Ferreira *et al.*, 1998), but absent in *C. subternata* (Brand, 2002) are not readily oxidised as demonstrated by their poor activity in antioxidant assays (Hubbe, 2000).

Mangiferin and/or isomangiferin were found to be the predominant phenolic compounds present in the unfermented and fermented aqueous extracts of the *Cyclopia* species used for this study. Brand (2002) isolated only mangiferin from unfermented *C. subternata*. The relatively high levels of mangiferin + isomangiferin is in agreement with previous findings by Joubert *et al.* (2002) where mangiferin was found to be the major compound present (1.04-3.61% of dry plant material) in unfermented *C. intermedia*, *C. sessiliflora* and *C. genistoides* with the isomangiferin present at lower levels (0.22-0.54% of dry plant material). Fermentation caused a decrease in the mangiferin + isomangiferin content of the aqueous

extracts. This could be attributed to the fact that mangiferin and isomangiferin (Hubbe, 2000; Sato *et al.*, 1992) are both good antioxidants and thus readily oxidised over other less active compounds. Mangiferin and isomangiferin have much higher antioxidant activity than hesperidin in the DPPH and superoxide radical scavenging assays (Hubbe, 2000), indicating it to be more susceptable to oxidation. However, the decrease in the levels of mangiferin + isomangiferin and hesperidin does not fully explain the decrease in total polyphenols with fermentation, indicating the presence of other compounds susceptible to oxidation and that polymerisation occurred. For instance the total polyphenol content of *C. subternata* soluble solids decreased from 32.41 to 17.16% with fermentation, while the apparent contribution of mangiferin and hesperidin decreased from 3.59 to 0.4 and 0.48 to 0.32, respectively. The unidentified peaks in *C. subternata* and *C. intermedia*, both with approximately the same absorbance as mangiferin, showing them to be major compounds, decreased substantially with fermentation.

These changes in phenolic composition of the DAE with fermentation caused both the ferric reducing and the $ABTS^{\bullet+}$ scavenging abilities of the total soluble solids of *Cyclopia* species to decrease. This is best explained by a decrease in total polyphenols ($r = 0.98$ for FRAP and $ABTS^{\bullet+}$) and flavanols ($r = 0.76$ for FRAP; $r = 0.74$ for $ABTS^{\bullet+}$). The decrease in ferric reducing and $ABTS^{\bullet+}$ scavenging abilities of *C. intermedia*, *C. subternata* and *C. sessiliflora*, with fermentation, was more substantial than for *C. genistoides*. The losses in mangiferin + isomangiferin and hesperidin only partially accounted for the reduced antioxidant activity after fermentation. For example the FRAP value of *C. intermedia* decreased by 1901, with 298 due to the decrease in mangiferin + isomangiferin and hesperidin. This indicates that other phenolic compounds, although present in small amounts, or polymers contribute substantially to the antioxidant activity. Compounds such as luteolin, eriodictyol and hesperetin isolated from fermented *C. intermedia* (Ferreira *et al.*, 1998) and unfermented *C. subternata* (Brand, 2002) have been proven to be good $ABTS^{\bullet+}$ scavenging antioxidants (Rice-Evans *et al.*, 1996) and to be far more effective as antioxidants than mangiferin and isomangiferin (Hubbe, 2000). In the same light mangiferin + isomangiferin, that made up 86% of the total polyphenols of unfermented *C. genistoides* accounted for *c.* 42% of the FRAP value. This indicates that direct extrapolations of activity versus content of a single compound to that of a mixture cannot be made. This apparent contribution decreased to 26% with fermentation. It was however, less active in the $ABTS^{\bullet+}$ assay, contributing *c.* 22 and 13% of the $ABTS^{\bullet+}$ scavenging activity of the unfermented and fermented total soluble solids of this

species, respectively. Hubbe (2000) found mangiferin to be only slightly less active than luteolin against the DPPH radical. Hesperidin, although one of the major phenolic metabolites of *C. intermedia* and *C. genistoides* (Table 2), had a negligible apparent contribution to both the FRAP and $ABTS^{\bullet+}$ values. The flavanone hesperidin only conforms to one of the three criteria deemed important for antioxidant activity by Bors *et al.* (1990) and Sichel *et al.* (1991), namely the 5-OH group in the A ring with the 4-oxo function in the C ring. Hesperidin, a glycoside is also methoxylated in the B-ring which has been shown to cause a decrease in antioxidant activity of flavanones (Shahidi & Wanasundara, 1992). These structural aspects account for the poor antioxidant activity observed for hesperidin. Mangiferin however, does not conform to any of the structural aspects of a "good" antioxidant, but still exhibits reasonable activity. The activity of the xanthone mangiferin can be attributed to the catecholic (6,7-dihydroxy) structure that is more susceptible to oxidation than the meta-disubstituted (1,3-dihydroxy ring), since a radical formed at the 6-OH may be delocalised to 7-OH and a radical formed at the 7-OH may be delocalised to 6-OH and the heterocyclic oxygen. In addition radicals formed at the 1-OH, 3-OH and 6-OH positions in the xanthone structure may also be delocalised to the carbonyl group [D. Ferreira, National Centre for Natural Product Research, University of Mississipi, United States of America, personal communication, 1999 (*as cited by* Hubbe, 2000)].

When the antioxidant activity determined in the FRAP and $ABTS^{\bullet+}$ assays was expressed in terms of total polyphenol content and not total soluble solids, an indication of the potency of the polyphenols in the extract was obtained. From this it became evident that the potency of the polyphenols also decreased with fermentation, suggesting that changes in the structure of compounds may effect their ferric reducing and $ABTS^{\bullet+}$ scavenging abilities more than their reactivity towards the Folin-Ciocalteu reagent even though all of these assays are based on oxidation-reduction reactions (Prior & Cao, 1999). Although the $ABTS^{\bullet+}$ and FRAP assays are conducted in different solutions, i.e. aqueous buffer and ethanol respectively, and function according to different mechanisms, i.e. reduction of ferric ion in the FRAP assay and scavenging of ABTS cation radicals in the $ABTS^{\bullet+}$ assay, these two assays were highly correlated ($r = 0.98$; $P < 0.0001$). Good correlation ($r = 0.79$; $P < 0.05$) between the antioxidant activity measured in the FRAP and $ABTS^{\bullet+}$ assays was also demonstrated for 18 rosehip extracts representing six taxa in the genus Rubus (Gao *et al.*, 2000). The use of both of these assays was prompted by the concerns of some authors who consider the ferric-reducing capacity as a pro-oxidant character (Plumb *et al.*, 1996). However, the good

correlation between these assays in the present study and other studies suggests that it may well be a suitable method of analysis.

The ability of the DAE of the *Cyclopia* species to inhibit lipid peroxidation in rat liver microsomes also decreased with fermentation, due to the decrease in total polyphenol content ($r = 0.79$) (Figure 20), and to a lesser degree, flavanol content ($r = 0.53$). Unfermented *C. sessiliflora* was the most potent inhibitor of lipid peroxidation. However, the DAE of unfermented *C. genistoides* with approximately the same total polyphenol content as unfermented *C. sessiliflora* performed the poorest of the unfermented species suggesting that qualitative differences and not quantitative differences in polyphenol content are important for the antioxidant activity. Fermentation did not affect the ability of *C. genistoides* to inhibit lipid peroxidation in rat liver microsomes. Hubbe (2000) showed that the aqueous extract of unfermented and fermented *C. genistoides* had the same efficacy against linoleic acid peroxidation in an emulsion. *Cyclopia genistoides* had a high concentration of mangiferin + isomangiferin even after fermentation. However, very high concentrations of mangiferin are required to inhibit lipid peroxidation. Mangiferin, a glucoside, is relatively polar and would therefore have a higher affinity for the hydrophilic phase, whilst the peroxyl radicals are generated in the lipophilic phase (Frankel & Meyer, 2000). The inhibitory potency of the total polyphenols of unfermented *C. sessiliflora* was the highest of the species and it retained this potency with fermentation, whereas for the other species, the inhibitory potency increased with fermentation. Loss of mangiferin + isomangiferin, second highest in *C. sessiliflora*, with fermentation, did not affect its lipid peroxidation inhibitory ability, confirming that these compounds are not very important in this assay. The combined effect of the lipophilic character in addition to structural conformation of compounds are important in protection of phospholipids in membranes against oxidation (Frankel & Meyer, 2000; Van Dijk 2000). Oxidation of a flavanone to its corresponding flavone and the removal of the sugar moiety of glycosides, such as hesperetin versus hesperidin, will enhance the affinity of compounds for the lipophilic phase (Mora *et al.*, 1990). Formation of the 2,3-double bond in the C ring imparts a more planar structure to a flavone such as luteolin in contrast to the puckered conformation of its flavanone, eriodictyol. According to Van Dijk *et al.* (2000) planar compounds favour association with the organised structure of the phospholipids within the membrane and would therefore be more effective as inhibitors of lipid peroxidation. It is important to consider lipid peroxidation as a complex process which can be influenced by flavonoids through different mechanisms, such as divalent metal chelation and free radical scavenging, possibly by donation of the phenolic hydrogen and formation of a flavonoid

radical which in turn reacts with free radicals, thus breaking the propogating chain (Mora *et al.*, 1990).

It has been stated that moderate consumption of *Camellia* teas (5 cups/day or an extract of about 11 g) may be a readily available, potential additional means of reducing the risk of some types of human cancer (Apostolides *et al.*, 1996). Reviews on the health promoting properties of *Camellia sinensis* teas show that they have been extensively investigated for their anticarcinogenic and antimutagenic potential (Wang *et al.*, 1989; Kuroda & Hara, 1999; Dufresne & Fanworth, 2001). In this study the antimutagenic potential of different *Cyclopia* species was shown to decrease with fermentation. This was in agreement with findings by Marnewick *et al.* (2000) and Standley *et al.* (2001) on the antimutagenic properties of South African herbal teas and Yen & Chen (1995) on the antimutagenic potential of *Camellia sinensis* (unfermented and fermented) extracts using various mutagens and tester strains.

Differences in antimutagenic potential between species is likely to be attributed to the varying phenolic composition, especially the flavonol + flavone content which was significantly correlated with antimutagenicity ($r = 0.60$; $P < 0.0001$). On the other hand, the flavanol content ($r = 0.12$; $P = 0.3945$) did not correlate with antimutagenicity indicating that the hydrophilic/lipophilic character of the compounds could be of importance. Unfermented *C. genistoides* contained predominantly mangiferin + isomangiferin, but since mangiferin was not promutagenic even at high concentrations the observed promutagenic response could be attributed to the remaining 12% total polyphenols or interaction between the polyphenols or possibly other compounds could play a role. Hesperidin was able to increase mutagenesis in the presence of 2-AAF, but the quantities present in *C. genistoides* were too low to account for all of the promutagenic activity. Hesperetin, the aglycone of hesperidin, and therefore of higher lipophilicity is a good antimutagen against aflatoxin B_1 in tester strain TA100 (Choi *et al.*, 1994). There is also the possibility of synergistic relationships between compounds (Moure *et al.*, 2001). The stimulation of mutagenicity by phenolic compounds has also been observed. Enhancement of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 2-amino-3,4,7,8-tetramethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx) mutagenesis was noted for *Camellia sinensis* teas (Stavric *et al.*, 1996). Studies testing the antimutagenic potential of quercetin and ellagic acid have found that by itself quercetin was mutagenic, but was also able to inhibit the activity of several food mutagens, while ellagic acid, a non-mutagenic polyphenolic substance increased the mutagenicity of Trp-P-1 and Trp-P-2 (Stavric *et al.*, 1990). Unfermented *C. intermedia* contains more hesperidin (Table 2) than *C. genistoides*, but it was not promutagenic. It appears that the other *Cyclopia* species contained compounds that

were able to perform better against metabolically activated 2-AAF in TA98. However, to fully understand the efficacy of the different species as antimutagenic agents it is important that in future these teas, also be evaluated against other mutagens using different tester strains and different plating techniques so that insight into the mechanisms of action may be obtained.

The observed promutagenic response or stimulation of mutagenicity by the polyphenolic compounds of *C. genistoides* and in particular, hesperidin in this study could be as a result of promotion of the phase I enzymes responsible for increased mutagenicity, since on its own the aqueous extract did not induce mutagenesis. Thus far a study investigating the mechanisms of antimutagenic actions against different mutagens in different tester strains of unfermented and fermented *C. intermedia* has been done (Marnewick *et al.*, 2000). It was postulated by Marnewick *et al.* (2000) that, amongst others, the antimutagenic activity of the aqueous extract of *C. intermedia* could be ascribed to either a direct interaction of the activated mutagenic metabolite with the tea polyphenols, or to an interaction between the different components of the tea extracts and the enzyme system catalysing the metabolic activation of the various promutagens. Through interfering with the enzyme system, cytochrome P_{450}-mediated activation of the carcinogen is inhibited and thus the production of genotoxic intermediates is impeded. It can be argued that the tea polyphenols are able to serve as electron acceptors thus directing the flow of electrons from NADPH away from cytochrome P_{450}, the terminal component in the electron-transport chain. Antioxidant activity of the aqueous extracts, obtained with all three assays showed no correlation with antimutagenic activity.

CONCLUSIONS

This study confirmed the importance of using different assays to evaluate the antioxidant activity of plant extracts such as those of *Cyclopia* species. Their relative importance as sources of antioxidants and the extent to which antioxidant activity changed with fermentation depended on the assay used. More insight into the phenolic composition of the species is needed to fully explain findings. This is underlined by the fact that the total polyphenols of *C. sessiliflora* retained its ability to protect against lipid peroxidation in rat liver microsomes while those of other species improved with fermentation. Antimutagenicity, especially the mechanisms involved and structure-activity relationships should be investigated in future to explain findings of this study. *Cyclopia genistoides* changed from being promutagenic to

antimutagenic with fermentation. This was linked to composition, e.g. hesperidin was shown to be promutagenic at high concentrations. On the other hand *C. intermedia* containing even more hesperidin than *C. genistoides* was not promutagenic, suggesting that synergistic effects could be prevalent in *C. genistoides*.

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CHAPTER 4

ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITY-GUIDED FRACTIONATION OF A METHANOL EXTRACT OF UNFERMENTED *CYCLOPIA INTERMEDIA*

ABSTRACT

Dichloromethane, ethyl acetate, methanol and water (sequential and non-sequential) extracts of unfermented *Cyclopia intermedia* were evaluated according to their *in vitro* antioxidant and antimutagenic activities. Scavenging of the synthetic 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$), reduction of a ferric-tripyridyltriazine complex and inhibition of Fe^{2+} induced lipid peroxidation using rat liver microsomes were used as measures of antioxidant activity. Antimutagenic activity against 2-acetylaminofluorene (2-AAF) induced mutagenesis of the extracts and fractions was determined in the *Salmonella typhimurium* antimutagenicity (AMES) assay (with metabolic activation and tester strain TA98). The methanol extract, affording the highest extraction (20 g 100 g^{-1} dry plant material) and total polyphenol (30 g 100 g^{-1} dry extract) yields, had the highest $ABTS^{\bullet+}$ scavenging (1851 μmol g^{-1}) and ferric reducing (3202 μmol g^{-1}) activities, while the ethyl acetate extract was the most effective against microsomal lipid peroxidation (65%) and 2-AAF induced mutagenesis (87%). The methanol extract inhibited lipid peroxidation by 46% and mutagenesis by 9%. Removal of the ethyl acetate soluble compounds from the plant material before methanol extraction improved its inhibitory effect against lipid peroxidation (52%) and mutagenesis (32%), indicating a possible antagonistic effect of these compounds in the non-sequential methanol extract. Activity-guided fractionation of the methanol extract was conducted by column chromatography on XAD, giving fractions A to F, followed by fractionation of E on C_{18}, giving fractions E_1 to E_6. The polar XAD fractions had higher $ABTS^{\bullet+}$ scavenging and ferric reducing activities than the non-polar fractions, while the latter exhibited better protective effects against microsomal lipid peroxidation and 2-AAF-induced mutagenesis. Mangiferin + isomangiferin were quantified as the major compounds present in fractions B to D and E_2, and hesperidin in

fractions E, F and E_4. The $ABTS^{\bullet+}$ scavenging and ferric reducing activities of the C_{18} fractions also decreased with increasing lipophilicity. Antimutagenic activity, however, was not affected by the lipophilicity of the fractions, with the most polar (E_1) and the least polar (E_6) fractions giving the highest activity. Fractions that exhibited promutagenic effects were not mutagenic against TA98 in the presence and absence of metabolic activation. Antioxidant and antimutagenic activities of the quantified major compounds only partially accounted for the antioxidant and antimutagenic effects of the fractions due to possible synergistic or antagonistic effects.

INTRODUCTION

Cyclopia intermedia, indigenous to the Southern Cape Province, is used to prepare an herbal tea free of caffeine (Greenish, 1881) and low in tannin (Terblanche, 1982). Traditional processing involves "fermenting" the stems, leaves and flowers in order to develop the sought-after taste and aroma profile of the beverage. The chemical oxidation that occurs during the "fermentation" process causes a reduction in the total polyphenols (Du Toit & Joubert, 1998) and a subsequent decrease in its antioxidant (Hubbe, 2000) and antimutagenic activities (Marnewick *et al.*, 2000). Fermented *C. intermedia* has been shown to contain a wide variety of phenolic compounds ranging from the major compounds, the xanthones mangiferin and isomangiferin and the flavanone hesperidin, to smaller amounts of glycosides from the flavonol, kaempferol, the isoflavones calycosin, fujikinetin, pseudobaptigen, formononetin and afrormosin, the flavanones hesperetin, eriodictyol and naringenin, the flavone luteolin and the coumestans medicagol, flemichapparin and sophoracoumestan B (Ferreira *et al.*, 1998; Kamara, 1999). Antioxidant activity in the form of $ABTS^{\bullet+}$ scavenging abilities (Rice-Evans *et al.*, 1996, Hubbe, 2000) and inhibition of lipid peroxidation (Mora *et al.*, 1990; Sato *et al.*, 1992; Hubbe, 2000) have been demonstrated for luteolin, naringenin, hesperetin and mangiferin, and eriodictyol, luteolin and mangiferin, repectively. Mangiferin is the major compound present in unfermented *C. intermedia* (*c.* 1.7 g 100 g^{-1} dry plant material) (Joubert *et al.*, 2002). This compound is well known for its cardiotonic, spasmolytic, diuretic, antimicrobial and antiviral actions (Peres *et al.*, 2000; Simova *et al.*, 1986). Luteolin, hesperetin and naringenin have been shown to be strong antimutagens (Choi *et al.*, 1994; Nakasugi *et al.*, 2000). These compounds are effective

against the mutagens, aflatoxin B_1 (AFB_1) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

Phenolic compounds play an important role in regulating elevated levels of oxidative stress that is associated with diseases such as cancer, arteriosclerosis, coronary heart disease and rheumatoid arthritis (Davies *et al.*, 1995; Moure *et al.*, 2001). Natural plant extracts rich in active compounds have huge potential in the nutraceutical and functional food markets as an increasing body of scientific evidence supports the role of phytopharmaceuticals in the prevention and treatment of diseases linked to oxidative stress (Sloan, 2002).

The present study investigated the potential of unfermented *C. intermedia*, as a source material for the preparation of a nutraceutical product rich in antioxidants and/or antimutagens. Different solvent extractions and activity-guided chromatographic fractionation based on the antioxidant and antimutagenic activities were employed in order to monitor the activities of the extracts and column fractions. Colorimetric methods and HPLC analysis were used to quantify total polyphenols and some of the major phenolic compounds of the extracts and fractions, respectively. Antioxidant activity of the extracts, column fractions and pure compounds was determined using different *in vitro* antioxidant assays while the antimutagenic activity was determined in the *Salmonella* mutagenicity assay.

MATERIALS AND METHODS

Column separation matrices and chemicals

Amberlite XAD-1180 polymeric beads were obtained from Merck (Darmstadt, Germany) and Bodesil-C_{18} (40 µm) from Varian (SMM Instruments, Cape Town). Pure phenolic standards, chemicals and water purification methods used, are described in Chapter 3. All other chemicals used were of analytical grade.

Thin layer chromatography (TLC)

Solvent extracts and column fractions were spotted on silica 60 F_{254} TLC plates supplied by Merck (Darmstadt, Germany). The TLC plates were developed in a chloroform:methanol:water:acetic acid (1100:720:160:20) solvent system. A *p*-anisaldehyde [purchased from Riedel de Haën (Darmstadt, Germany)] solution consisting of 0.5 g *p*-

anisaldehyde, 85 mL methanol, 10 mL acetic acid and 5 mL sulphuric was used as spray reagent. Colour development was achieved by heating the plates at 100°C for 10 minutes. Extracts and fractions were screened for their hydrogen donating abilities by spraying with a 0.4 mM 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) [purchased from Sigma Chemical Co (St Louis, USA)] in methanol and allowing the colour reaction to develop in the dark for 1 hour according to the method of Soler-Rivas *et al.* (2000). Spots that change the $DPPH^{\bullet}$ solution from purple to bright yellow have hydrogen donating abilities.

Owing to the instability of the colour reactions formed through the visualisation processes TLC plates were captured by scanning and transferred to electronic format immediately after visualisation.

Plant material

Approximately 80 kg of *C. intermedia* was harvested in February 2001 from a plantation established in October 1999 at the ARC experimental farm, Helderfontein, Stellenbosch. The plant material was dried at 30°C for 12 hours in a drying tunnel (Decon Humidifier, Continental Fan Works CC., Cape Town, South Africa) to a moisture content < 10% (wet basis) before being pulverised in a Retch rotary mill. The finely ground material was stored in plastic containers in the dark.

Solvent extracts

Solvent extracts of the pulverised plant material were prepared through both sequential and non-sequential extraction using dichloromethane (CH_2Cl_2), ethyl acetate (EtAc), methanol (MeOH) and water in order of increasing polarity. For sequential extraction 50 g of the plant material was extracted with 100 mL CH_2Cl_2 in a separating funnel for 5 minutes and filtered through a Whatman No. 4 filter (Whatman International Ltd., Maidstone, England). The plant material was re-extracted twice with CH_2Cl_2 (100 mL). The filtrates were pooled and dried *in vacuo* at 40°C on a Büchi rotovap (Lasec, South Africa). The extracted plant material was subsequently extracted as described above with EtAc followed by MeOH and finally deionised water. The filtrates from the EtAc and MeOH extractions were dried *in vacuo* on a Büchi rotovap, while the aqueous extract was frozen at -20°C in plastic trays (170 x 115 x 30mm) before freeze-drying in an Atlas pilot-scale freeze-drier (Denmark model, Copenhagen, Denmark, 40°C shelf temperature). The non-sequential extraction involved extracting 50 g of the plant material firstly with 3 x 100 mL dichloromethane for 5 minutes

in a separating funnel and subsequently with either EtAc, MeOH or water (3 x 100 mL) and dried in the same manner as for the sequential extracts. The non-sequential and sequential extractions were performed in triplicate.

Preparation of methanol extract

The pulverized plant material (500 g) was extracted in a 2 L Erlenmeyer flask with 2 L CH_2Cl_2 on a magnetic stirrer for 20 hours and filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The plant residue was extracted further with 2 x 2 L CH_2Cl_2 in the same manner prior to extraction with MeOH (2x2L). The CH_2Cl_2 extract was discarded and the pooled MeOH extracts were dried *in vacuo* on a Büchi rotovap at 40°C. The extraction procedure was repeated with another 500 g of plant material. The dried MeOH extract was ground to a fine dust in a Fritsch pulverisette ball mill (Labotec, Johannesburg) to obtain a homogenous sample.

XAD column chromatography

The methanol extract was crudely fractionated on XAD-1180 polymeric beads. An open glass column (650 x 45 mm) was packed with XAD (previously washed with distilled water to remove chloride ions) suspended in 15% MeOH:H_2O. The MeOH extract (20 g) was dissolved in 40 mL 15% MeOH:H_2O and allowed to pre-adsorb to 80 g XAD overnight. This slurry was applied to the top of the column and the beaker rinsed with 40 mL 15% MeOH:H_2O which was transferred onto the column. The initial 45 mL that eluted from the column during sample application was discarded. The column was run at a flow rate of 20 mL min^{-1} and sample collection (400 mL fractions) began after collection of the pre-fraction (200 mL). A stepwise elution consisting of 2 L each of 15% MeOH:H_2O, 30% MeOH:H_2O, 50% MeOH:H_2O, 80% MeOH:H_2O and 100% MeOH was run at room temperature. The fractions (f_1 –f_{26}) were spotted on silica F_{254} TLC plates and the components visualised.. The fractions with corresponding bands were pooled into 6 fractions (A to F):

A = Pre-fraction + $f_1 - f_3$

B = $f_4 - f_8$

C = $f_9 - f_{13}$

D = $f_{14} - f_{16}$

E = $f_{17} - f_{22}$

F = $f_{23} - f_{26}$

The fractionation procedure was repeated on another 20 g of MeOH extract to obtain enough material for the C_{18} column fractionation step. The XAD packing material was regenerated between fractionation procedures by rinsing thoroughly with hexane, iso-propanol and methanol.

Reversed-phase C_{18}

Fraction E (XAD) was fractionated further on reversed-phase Bondesil-C_{18} (40μm). A C_{18} closed glass column (360 x 25 mm) was prepared in methanol and light was excluded with a black material jacket. The solvent composition was slowly changed to 50% MeOH:H_2O for equilibration. The sample (304.7 mg) was suspended in 1 mL 50% MeOH:H_2O by ultra-sonication for 5 minutes. The insoluble residue (21.9 mg) present in the sample was filtered out with a 13 mm 0.45 μm HV filter from Millipore (Ireland). The residue was washed with 6 mL 50% MeOH:H_2O to remove any remaining soluble components. The filtrate was applied to the column by reversing the flow on a Gilson minipuls 3 peristaltic pump (Lasec, South Africa) set at *c.* 0.5 mL min^{-1}. After sample application the column was run isocratically at a flow rate of 0.472 mL min^{-1} on 200 mL 50% MeOH:H_2O for 423.73 minutes. Collection of the 80 mL pre-fraction began on application of the sample to the column after which 7.55 mL (16 min) fractions were collected on a Gilson FC 203B micro fraction collector (Lasec, South Africa). Thereafter the column was eluted as follows: 500 mL 50% MeOH:H_2O to 80% MeOH:H_2O linear gradient for 1059.32 min; 180 mL of 80% MeOH:H_2O for 381.35 min; and 250 mL 100% MeOH for 529.66 min. A total of 139 x 7.55 mL fractions were collected, spotted on silica F_{254} TLC plates and developed. Visualisation with *p*-anisaldehyde allowed for fractions with corresponding bands to be pooled into 6 fractions (E_1 to E_6):

E_1 = Pre-fraction + $f_1 - f_{10}$

E_2 = $f_{11} - f_{16}$

E_3 = $f_{17} - f_{32}$

E_4 = $f_{33} - f_{52}$

E_5 = $f_{53} - f_{73}$

E_6 = $f_{74} - f_{139}$

This fractionation procedure was repeated four times under identical condition and corresponding fractions pooled in order to obtain enough material for testing of antioxidant

and antimutagenic activities. The C_{18} packing material was regenerated between fractionation procedures by rinsing thoroughly with hexane, iso-propanol and methanol.

Total polyphenol content of solvent extracts and fractions

The total polyphenol content was determined according to Singleton & Rossi (1965) as described in Chapter 3. A stock solution of between 0.15 and 4.7 mg mL^{-1} of the solvent extracts and fractions dissolved in DMSO was prepared for the determination of their total polyphenol content. The gallic acid equivalents of the pure standards mangiferin, hesperidin, hesperetin, luteolin, naringenin, formononetin and eriodictyol, were determined by reaction with the Folin-Ciocalteu reagent. The pure compounds, including the gallic acid, were diluted in DMSO to give dilution series with absorbance readings of between 0.2 and 0.8.

HPLC analysis of XAD and C_{18} fractions

Stock solutions of the samples (20 mg mL^{-1}) were prepared in DMSO through ultra-sonication for 5 min and filtration through a 13 mm 0.45 μm HV filter from Millipore (Ireland) for the quantification of mangiferin + isomangiferin, hesperidin, hesperetin, luteolin, naringenin, eriodictyol and formononetin content. HPLC separations were performed as described in Chapter 3. Peaks were tentatively identified according to retention times of the authentic standard phenolic standards (see addendum A for chromatogram with retention times). The peak area, obtained through valley to valley integration, was used to calculate the concentration of the compounds in the sample. For the standard curves, concentration gradients of 0.3-9.0 μg 10 μL^{-1} of mangiferin (for mangiferin + isomangiferin) and 0.07-2.00 μg 10 μL^{-1} for the other compounds were prepared in DMSO and filtered through a 13 mm 0.45 μm HV filter. Samples (10 μL) were injected automatically and the absorbance measured at 280 nm.

Radical scavenging and ferric reducing ability as a measure of total antioxidant activity

The $ABTS^{\bullet+}$ scavenging activity of the solvent extracts and MeOH fractions was determined using the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical cation decolourisation assay (Re *et al.*, 1999) as described in Chapter 3. Stock solutions of the solvent extracts (0.15-4.2 mg mL^{-1}) and fractions (0.1-0.9 mg mL^{-1}) were prepared in DMSO and dissolved with the aid of ultra-sonication for 5 min. The stock solution was diluted to give between 20-80% scavenging of $ABTS^{\bullet+}$ at 734 nm.

The ferric reducing ability of extracts and column fractions of the MeOH extract was determined according to the manual procedure of the ferric reducing antioxidant power assay (FRAP) (Benzie & Strain 1999) as described in Chapter 3. Stock solutions of the solvent extracts and fractions were prepared as described for the $ABTS^{\bullet+}$ assay and diluted in order to give a reaction mixture with an absorbance of between 0.2-0.8 at 593 nm.

The $ABTS^{\bullet+}$ and FRAP assays were also performed on the pure compounds, mangiferin, hesperidin, hesperetin, luteolin, naringenin, eriodictyol and formononetin. The compounds were dissolved in DMSO to a reaction mixture concentration of 2.6-10.4 μM (mangiferin) and 6.2-31.2 μM (hesperidin, hesperetin, luteolin, naringenin, eriodictyol and formononetin) for the $ABTS^{\bullet+}$ assay, and 5.6-45.1 μM (mangiferin) and 28.2-225.5 μM (hesperidin, hesperetin, luteolin, naringenin, eriodictyol and formononetin) for the FRAP assay.

Rat liver microsomal lipid peroxidation

Inhibition of lipid peroxidation in rat liver microsomes by the solvent extracts and column fractions was determined according to a modified version of the method by Yen & Hsieh (1998) as described in chapter 3. Stock solutions of the solvent extracts (14 mg mL^{-1}) and fractions (30 mg mL^{-1}), prepared in DMSO and dissolved with the aid of ultra-sonication for 5 minutes, were diluted to give between 20-80% inhibition.

A dose response of the pure compounds, mangiferin, hesperidin, hesperetin, luteolin, naringenin, eriodictyol and formononetin was tested, and comparison between compounds was made at a concentration at 300 μM in the reaction mixture.

Antimutagenic activity (*Salmonella typhimurium* plate incorporation test)

The antimutagenic activity of the solvent extracts and column fractions against 2-AAF induced mutagenesis against tester strain TA98 in the presence of S-9 mix was determined using the *S. mutagenicity* plate-incorporation test by Maron & Ames (1983) as described in chapter 3. A dose response effect was tested at concentrations of between 0.1-8 mg $plate^{-1}$, depending on the degree of antimutagenic response obtained. All solvent extracts and fractions were also tested at a concentration of 1 mg $plate^{-1}$ for comparative purposes. Stock solutions of 0.1-8 % were prepared in DMSO and dissolved with the aid of ultra-sonication for 5 minutes.

The antimutagenic activity of the pure compounds, mangiferin, hesperidin, hesperetin, luteolin, naringenin, eriodictyol and formononetin, was determined in a dose response manner (0.25-8 mg plate^{-1}) and comparison between compounds was made at 300 μM.

Statistical analysis

One way analysis of variance followed by Student's t-LSD (SAS Release version 6.12) was performed on the means to determine significant differences between solvent extracts (sequential and non-sequential extraction with different solvents) at a significance level of 0.05. All the means were correlated and Pearson's correlation coefficient obtained.

RESULTS

Antioxidant and antimutagenic activities of pure compounds

The reactivity of the flavonoids towards the Folin-Ciocalteu reagent is given in Table 1. Eriodictyol, the most reactive, gave a stronger colour reaction than gallic acid requiring 0.58 μM to give the same absorbance as 1 μM gallic acid. Hesperidin (1.1 μM) gave a similar reaction towards the Folin-Ciocalteu reagent as gallic acid (1 μM). Formononetin showed no reaction at the concentrations tested.

Ranking of the phenolic compounds according to antioxidant activity differed depending on the assay used (Table 1). For $ABTS^{\bullet+}$ scavenging activity the following order relative to Trolox was observed: mangiferin > Trolox > luteolin > eriodictyol > hesperidin > hesperetin > naringenin. Formononetin did not exhibit $ABTS^{\bullet+}$ scavenging activity at the concentrations tested, but displayed ferric reducing abilities and performed better than naringenin. However, it was not comparable to mangiferin, luteolin, hesperidin, hesperetin or eriodictyol. In the FRAP assay the compounds could be ranked as follows: mangiferin = eriodictyol > luteolin > hesperidin > hesperetin > formononetin > naringenin (Table 1). The ranking changed drastically when their ability to inhibit Fe^{2+} induced lipid peroxidation is considered: eriodictyol > luteolin > hesperetin > mangiferin > hesperidin > naringenin. Overall luteolin and eriodictyol performed well in all of the assays, while naringenin performed poorly. Formononetin was also not effective as inhibitor of lipid peroxidation at the concentration range (0.09-1 mg mL^{-1} in the reaction mixture) tested. Mangiferin, the

most active in both $ABTS^{\bullet+}$ and FRAP assays, was moderately effective at inhibiting lipid peroxidation (47%). Hesperidin, with a poor response in the lipid peroxidation assay, was moderately effective in the $ABTS^{\bullet+}$ and FRAP assays (Table 1).

Of the seven compounds tested for antimutagenic activity (Table 1), luteolin was the only compound that showed an appreciable level of activity. At an equal molar concentration mangiferin and hesperetin were only able to inhibit 2-AAF induced mutagenesis by 8-9%, while the remaining compounds gave promutagenic responses from 9-325%. These compounds however, did not show a mutagenic response in TA98 when tested at the same concentrations in the mutagenicity assay in the presence and absence of S9 (Table 2).

Yield and total polyphenol content of solvent extracts

Solvent extracts spotted on Silica F_{254} TLC plates (Figure 1) showed that extraction of the phenolic compounds was reproducible and that the solvents used did not extract the same mixture of compounds. Although there was overlap between the compounds extracted with EtAc and MeOH, and MeOH and H_2O, the concentration of individual compounds (based on intensity of the individual spots) in these solvents differed.

The yield of total polyphenols extracted differed significantly between solvents (Table 3). Non-sequential H_2O extraction gave the highest yield (25%, with 27% total polyphenols). The yield decreased to 20% with MeOH as solvent, but the total polyphenol content of the extract increased to 32%. Prior extraction of the plant material with EtAc did not significantly reduce the yield or total polyphenol contents of the MeOH extract. Ethyl acetate was not effective at extracting the total polyphenols from *C. intermedia*, demonstrated by its low yield and total polyphenol content. The CH_2Cl_2 extract contained almost no total polyphenols (1.11%) (table 3). HPLC quantification of the major phenolic compounds present in the methanol extract that was fractionated on XAD revealed it to contain 5.99 g 100 g^{-1} dry fraction mangiferin + isomangiferin and 1.13 g 100 g^{-1} dry fraction hesperidin (Figure 2).

Antioxidant and antimutagenic activity of solvent extracts

The antioxidant activity of the sequential solvent extracts in the $ABTS^{\bullet+}$ and FRAP assays increased significantly ($P < 0.05$) with the increasing polarity of the solvent used: CH_2Cl_2

Table 1. Relative antioxidant $ABTS^{\bullet+}$ decolourisation (TEAC), ferric reducing antioxidant power (FRAP) and Fe^{2+}induced rat liver microsomal lipid peroxidation (TBARS) assays) and antimutagenic activities [*S. typhimurium* plate incorporation assay (Ames)] of pure phenolic compounds, as well as their reactivity towards the Folin-Ciocalteu reagent (TP).

	Free OH substitution & Glycosylated position	TP[a]	TEAC[b]	FRAP[c]	TBARS[d]	Ames[e]
Mangiferin	2-β-*D*-glucopyranosyl	0.74	0.62	0.26	48.64	313.01 (7.66)
Luteolin	5,7,3',4'	0.62	1.03	0.61	69.99	33.31 (86.45)
Hesperidin	5,7-rutinose,3',4'-Ome	1.1	4.13	0.93	16.74	448.93 (-8.85)
Hesperetin	5,7,3',4'-OMe	0.73	4.80	1.24	66.62	326.95 (8.99)
Naringenin	5,7,4'	0.77	23.97	96.95	16.33	1217.36 (-325.88)
Eriodictyol	5,7,3',4'	0.58	1.26	0.26	71.25	545.92 (-60.06)
Formononetin	7, 4'-OMe	-	-	11.48	0	715.32 (-109.58)

[a] Expressed as μM required to give the same absorbance as 1 μM gallic acid. Values calculated from a standard curve of a dilution series with 0.2-0.8 absorbance ($0.97 < r < 0.99$).
[b] Expressed as μM required to give the same % inhibition as 1 μM Trolox. Values calculated from a standard curve of a dilution series with 20-80 % inhibition ($0.87 < r < 0.99$).
[c] Expressed as μM required to give the same absorbance as 1 μM $FeSO_4 \bullet 7H_2O$. Values calculated from a standard curve of a dilution series with 0.2-0.8 absorbance ($0.96 < r < 0.99$).
[d] Percentage inhibition of Fe^{2+} induced lipid peroxidation at a concentration of 300 μM in the reaction mixture.
[e] Number of revertants induced by 300 μM in the reaction mixture with metabolically activated 2-acetylaminofluorene (5 μg $plate^{-1}$) in TA98. Values in brackets are the corresponding % inhibition. Revertants for the positive control were 365.62 ± 33.7 and for the negative control 29.13 ± 9.8.

Table 2. Effect of pure compounds[a] on the number of revertants in TA98 with and without metabolic activation in the *S. typhimurium* plate incorporation assay.

	Revertants[b]									
	0.25 mg plate^{-1}		0.5 mg plate^{-1}		1 mg plate^{-1}		2 mg plate^{-1}		4 mg plate^{-1}	
	+S9	*-S9*	*+S9*	*-S9*	*+S9*	*-S9*	*+S9*	*-S9*	*+S9*	*-S9*
Hd	38	32	44	31	34	25	37	18	34	25
Ht	36	31								
N	42	26	35	33	34	34				
E	40	37	37	33						
F	36	37	46	34	56	47				
- Control[c]					34.4 ± 5.03					
+ Control[d]					480.8 ± 11.54					

[a] Hesperidin (Hd), hesperetin (Ht), naringenin (N), eriodictyol (E) and formononetin (F).

[b] Number of revertants with and without the addition of S9 in TA98 of pure compounds and fractions exhibiting promutagenic effects. Each value represents the mean of analysis done in triplicate except for the negative and positive controls that had 5 replicates.

[c] The number of spontaneous revertants ± S.D. in TA98.

[d] The number of revertants ± S.D. induced by 2-acetylaminoflourene (5 μg plate^{-1}) in TA98 with metabolic activation.

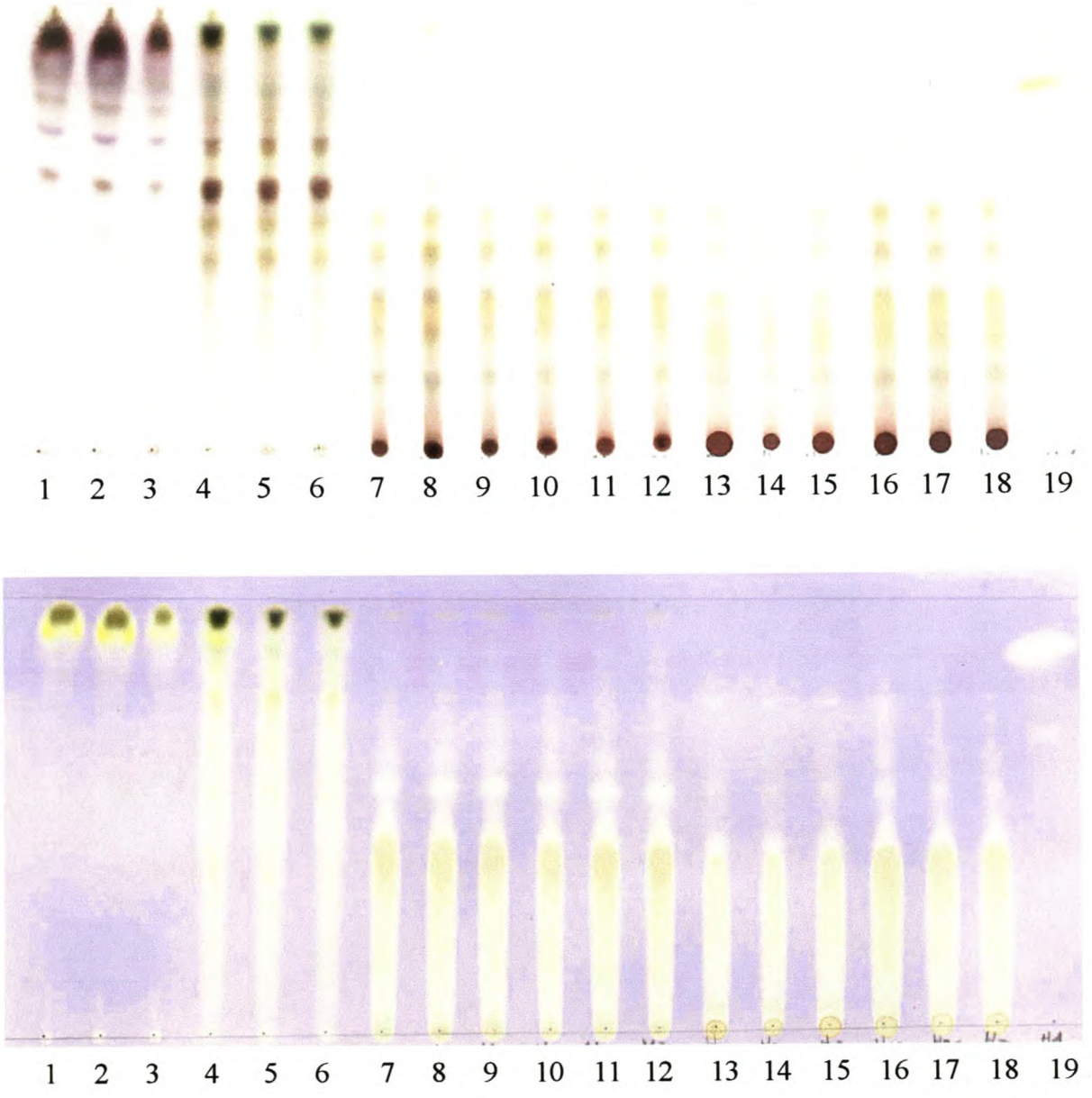

Figure 1. Sequential and non-sequential solvent extracts (done in triplicate) of unfermented *Cyclopia intermedia* spotted (at equal concentration) on silica F_{254} TLC plates and visualised with *p*-anisaldehyde (top) and DPPH$^{\bullet}$ in methanol (bottom). Spots with hydrogen donating abilities cause the DPPH$^{\bullet}$ spray reagent to change from purple to yellow. Dichloromethane extracts (lane 1-3), ethyl acetate extracts (lane 4-6), sequential methanol extracts (lane 7-9), non-sequential methanol extracts (lane 10-12), sequential aqueous extracts (lane 13-15) and non-sequential aqueous extracts (lane 16-18), hesperetin (lane 19).

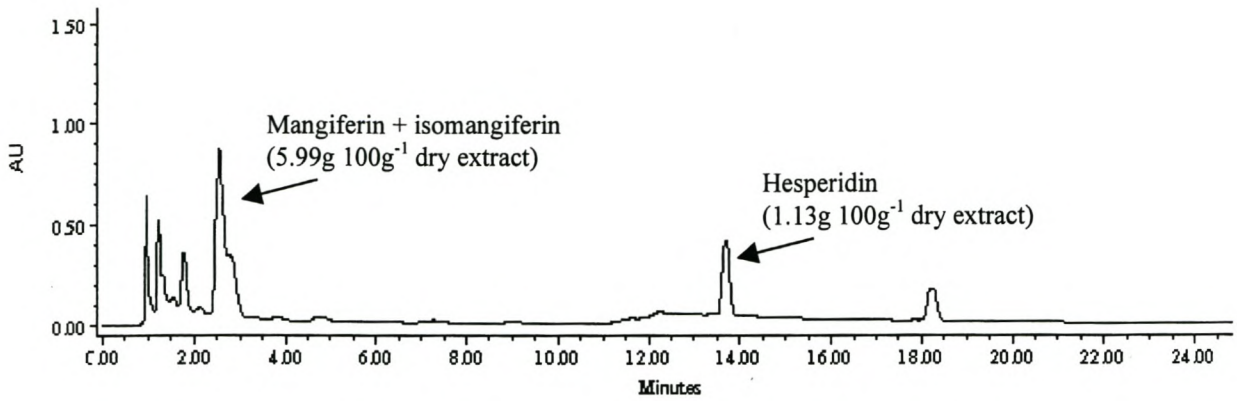

Figure 2. Chromatogram of the dry methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) with quantified major compounds mangiferin + isomangiferin (5.99 g 100 g^{-1} dry extract) and hesperidin (1.13g 100 g^{-1} dry extract). HPLC analysis was carried out on a Zorbax SB-C_{18} (3.5 µm particle size; 150 x 3 mm) column under gradient conditions; mobile phase, acetic acid:acetonitrile; flow rate, 1 mL min^{-1}; detection, 280 nm; column temperature, 30°C.

<<< EtAc << MeOH (Table 3). However, the water extract was less effective than the methanol extract. The dichloromethane and EtAc extracts were not effective in scavenging $ABTS^{\bullet+}$ and reducing Fe^{3+}. In both the $ABTS^{\bullet+}$ and FRAP assays the sequential MeOH extract had the highest activity (1887 and 3181 μmol g^{-1}, respectivey). However, no significant ($P > 0.05$) difference existed between the non-sequential MeOH and H_2O extracts when comparing their $ABTS^{\bullet+}$ scavenging ability, but the MeOH extract (3181 μmol g^{-1}) had a significantly ($P < 0.05$) higher ferric reducing ability than the water extract (2267 μmol g^{-1}). $ABTS^{\bullet+}$ scavenging and ferric reducing activities were well correlated with each other ($r = 0.98$; $P < 0.0001$) (Table 4).

The $ABTS^{\bullet+}$ scavenging and ferric reducing activities of the different solvent extracts were well correlated with total polyphenol content ($r \geq 0.98$; $P < 0.0001$) (Table 4). The polyphenol potency of the extracts, expressed in terms of total polyphenol content, was therefore calculated. Methanol extraction gave total polyphenols with a slightly lower potency towards $ABTS^{\bullet+}$ than the H_2O and CH_2Cl_2 extracts, while the potency of EtAc soluble polyphenols was the lowest. According to the FRAP assay the polyphenol potency (Table 3) of the EtAc, MeOH and H_2O extracts was not significantly ($P>0.05$) different, but substantially more potent than those of dichloromethane.

Solvent extracts performing well in the $ABTS^{\bullet+}$ and FRAP assays did not necessarily show an ability to inhibit Fe^{2+} induced rat liver microsomal lipid peroxidation (extracts differed significantly from one another based on the solvent used and not on extraction method) (Table 3). The EtAc extract provided the greatest protection against lipid peroxidation (65% inhibition) followed by the sequential MeOH extract (51% inhibition). Aqueous extraction afforded compounds with the lowest protective effect against lipid peroxidation (30% inhibition). Unlike the correlation obtained with total polyphenol content for both the $ABTS^{\bullet+}$ scavenging and ferric reducing activities, the ability of the solvent extracts to inhibit lipid peroxidation could not be significantly correlated with total polyphenol content ($r = -0.54$; $P = 0.2231$) nor could lipid peroxidation be significantly ($P > 0.05$) correlated with the antioxidant activity measured in the $ABTS^{\bullet+}$ and FRAP assays (Table 4).

When considering antimutagenicity (Table 3) EtAc extraction afforded compounds with the highest antimutagenic properties (87%). Dichloromethane extraction also yielded compounds with high antimutagenic properties against 2-AAF (76% inhibition). Non-sequential extraction yielded extracts with significantly less activity than their sequential

counterparts (9 and 35% inhibition for MeOH and H_2O, respectively, as opposed to 32 and 50% inhibition for these solvents with sequential extraction). The ability of the extracts to inhibit mutagenesis at different concentrations (Figure 3) showed that the CH_2Cl_2 extract was able to provide protection (30% inhibition) at the lowest concentration (0.1 mg plate^{-1}) and over the greatest concentration range (0.1-1 mg plate^{-1}). EtAc only provided protection once the concentration was increased from 0.1 to 0.25 mg plate^{-1}, while for the MeOH and H_2O extracts, concentrations had to be increased to 1 mg plate^{-1} before any protective effects could be observed. The difference in activity of the extracts obtained through sequential and non-sequential extraction was more evident at the lower concentration tested (1 mg plate^{-1}) with non-sequential MeOH and H_2O extracts having a poorer antimutagenic activity (14 and 32% as opposed to 46 and 52% inhibition, respectively).

The antioxidant activity of the extracts measured in the ABTS$^{\bullet+}$ and FRAP assays correlated well with antimutagenic activity against 2-AAF in TA98 ($r \geq 0.88$; $P < 0.0001$). The ability of the extracts to inhibit lipid peroxidation did not show any correlation with antimutagenic activity (Table 4).

Polyphenol composition of XAD fractions (A-F)

Fractions f_1-f_{26} were pooled according to the distribution of compounds after separation on silica F_{254} TLC plates (Figure 4). The TLC plate of the pooled fractions A-F, sprayed with DPPH$^{\bullet}$, shows that all fractions displayed antioxidant activity (Figure 5).

Fractions A and D had the highest yield and fractions C and Dtotal polyphenol content, respectively (Table 5). Taking yield into consideration fraction C contained 8% of the total polyphenols present in the original MeOH extract. Fractions A, B and F contained very little of the total polyphenols (<1.8%) of the MeOH extract. Fraction E had a relatively good fraction yield (18 g 100 g^{-1} dry extract) and, after fraction C, yielded the highest total polyphenols (5%). This extract exhibited both moderate ABTS$^{\bullet+}$ scavenging and ferric reducing activities that were half that of the most active fractions C and D, but its ability to inhibit lipid peroxidation was equal to these fractions. It exhibited excellent antimutagenic effects (75%) in comparison to fractions C (6%) and D (27%).

HPLC chromatograms of fraction A-F are given in Figure 6. Apart from compounds, mangiferin + isomangiferin and hesperidin, smaller peaks that did not elute at retention times given by the standards could also be observed. The concentration of these compounds was below the detection limit of the current method and could therefore, not be

Table 3. Yield, total polyphenol content (TP), radical scavenging ability (TAA), ferric reducing ability (FRAP), ability to inhibit Fe^{2+} induced rat liver microsomal lipid peroxidation (TBARS) and antimutagenic activity (Ames) in the *S. typhimurium* antimutagenicity assay of solvent extracts of unfermented *C. intermedia*[a].

Solvent[h]	Yield[b]	TP[c]	TAA[d]	FRAP[e]	TBARS[f]	Ames[g]
Dichloromethane extract	4.66d ± 0.54	1.11e ± 0.19 (0.05)	76.67d ± 8.68 (69.68a ± 4.14)	275.03e ± 18.5 (251.33a ± 27.12)	45.88b ± 1.39	79.40d ± 29.33 (76.1)
Ethyl acetate extract	0.94e ± 0.10	11.33d ± 0.69 (0.11)	446.76c ± 42.57 (39.39c ± 2.39)	1037.95d ± 97.72 (9162b ± 7.77)	65.02a ± 4.8	43.20e ± 4.21 (86.9)
Methanol extract	19.82b ± 0.42	31.54a ± 1.51 (6.25)	1887.45a ± 66.95 (59.88b ± 0.98)	3181.01a ± 140.59 (1009b ± 1.74)	51.46b ± 1.59	224.33b ± 21.36 (32.4)
Non-sequential methanol extract	20.20b ± 0.58	30.56a ± 0.54 (6.17)	1815.89a ± 50.23 (59.43b ± 1.96)	3223.84a ± 40.68 (1055b ± 0.99)	46.14b ± 1.30	302.47a ± 30.30 (8.9)
Water extract	10.08c ± 0.52	22.21c ± 1.85 (2.24)	1519.46b ± 68.23 (68.61a ± 3.98)	2267.41c ± 174.49 (1021b ± 0.97)	29.74c ± 2.77	168.47c ± 5.03 (49.3)
Non-sequential water extract	25.24a ± 0.26	27.75b ± 0.67 (7.00)	1866.08a ± 86.92 (67.22a ± 1.98)	2927.03b ± 86.14 (1055b ± 0.56)	30.68c ± 5.16	217.60b ± 5.92 (34.5)

[a] Values are the mean ± S.D. of three repeats with analyses done in triplicate (for the Ames assay 5 replicates were used). Means within the same assay followed by the same letter are not significantly different ($P < 0.05$).

[b] Expressed as g 100 g^{-1} plant material.

[c] Expressed as g gallic acid equivalents 100 g^{-1} extract and gallic acid equivalents 100 g^{-1} plant material in brackets.

[d] Total antioxidant activity expressed as µmol Trolox equivalents g^{-1} extract and polyphenol potency expressed as µmol Trolox g^{-1} gallic acid equivalents in brackets

[e] Ferric reducing antioxidant power expressed as µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} extract and polyphenol potency expressed as µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} gallic acid equivalents in brackets.

[f] Percentage inhibition of lipid peroxidation measured as thiobarbituric reactive substances on an equal extract mass basis.

[g] Revertants $plate^{-1}$ and percentage inhibition in brackets against 5 µg 2-AAF induced mutagenesis in TA98 at 1 mg $plate^{-1}$ extract. The mean number of 2-AAF induced revertants was 332.42 ± 15.75 and the frequency of spontaneous reversion was 29.27 ± 4.14.

[h] Extraction using dichloromethane, ethyl acetate, methanol and water took place both sequentially (in order of polarity) and non-sequentially (with dichloromethane extraction prior to extraction with either ethyl acetate, methanol or water).

Table 4. Overall correlation coefficients between antioxidant activities[a], antimutagenic activity[b] and total polyphenol content[c] of solvent extracts of unfermented *C. intermedia.*

	ABTS[a]	FRAP[a]	LP[a]	AMES[b]
TP[c]	0.98[d] (<0.0001)[e]	0.99 (<0.0001)	-0.54 (0.2231)	0.92 (<0.0001)
ABTS		0.98 (<0.0001)	-0.68 (0.0538)	0.94 (<0.0001)
FRAP	0.98 (<0.0001)		-0.59 (0.1611)	0.94 (<0.0001)
LP	-0.68 (0.0538)	-0.59 (0.1611)		-0.65 (0.0805)
AMES	0.94 (<0.0001)	0.94 (<0.0001)	-0.65 (0.0805)	

[a] Radical scavenging ability in the $ABTS^{\bullet+}$ assay, ferric reducing ability in the ferric reducing antioxidant power (FRAP) assay and ability to inhibit Fe^{2+}-induced rat liver microsomal lipid peroxidation (LP).

[b] Antimutagenic activity (AMES) of 1 mg $plate^{-1}$ solvent extract towards 2-acetylaminofluorene (5 µg $plate^{-1}$) using TA98 in the *S. typhimurium* antimutagenicity assay.

[c] Total polyphenol content (TP) of the extract using Folin-Ciocalteu reagent and gallic acid as standard.

[d] Pearson's correlation coefficient.

[e] Probability

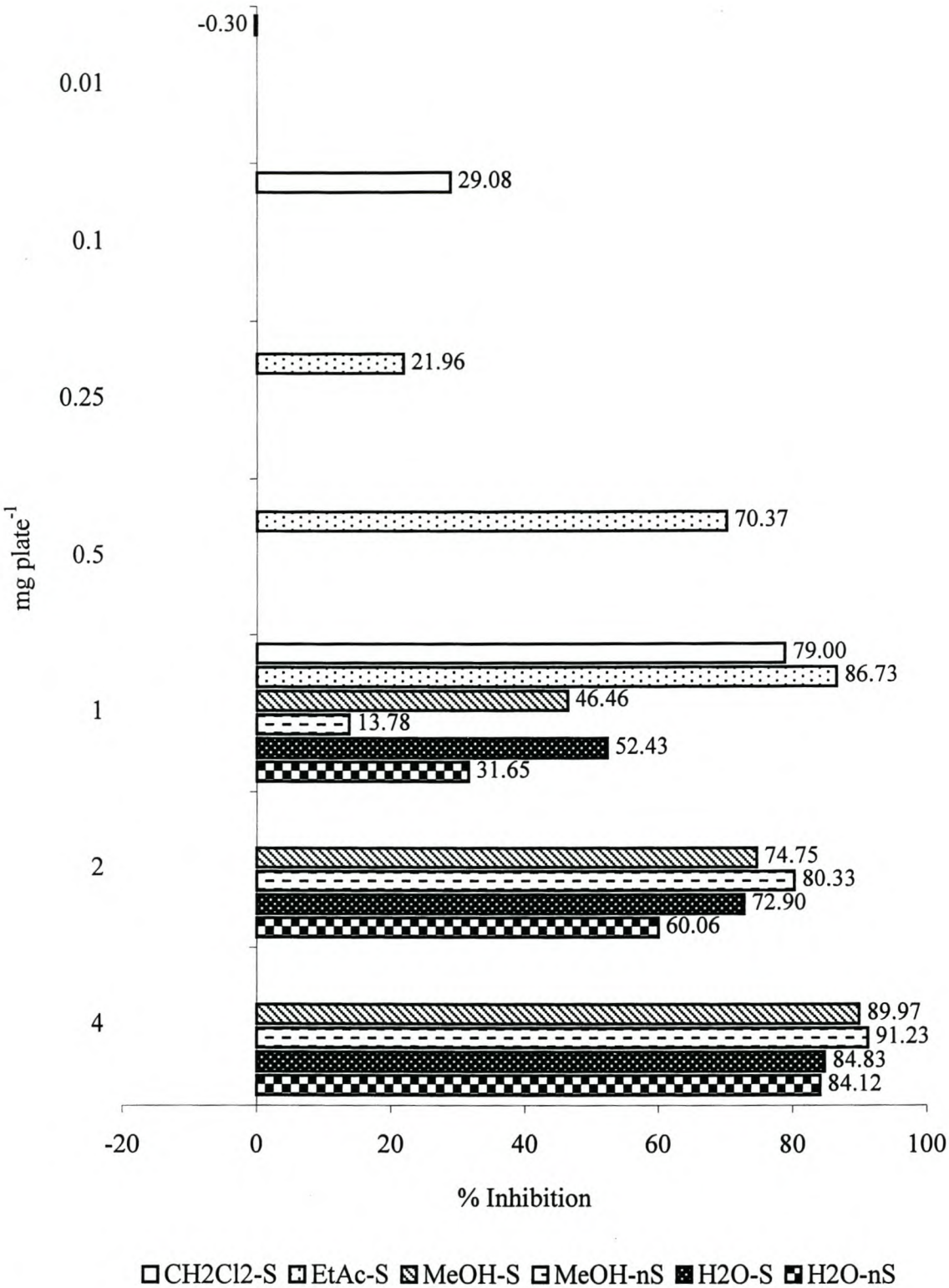

Figure 3. Antimutagenic activity of sequential (S) dichloromethane (CH2Cl2-S), ethyl acetate (EtAc-S), methanol (MeOH-S) and water (H_2O-S) and non-sequential (nS) methanol and water solvent extracts of unfermented *C. intermedia* against 2-acetylaminofluorene in the *S. typhimurium* plate-incorporation assay with TA98 as tester strain. Values are the mean ± S.D. % inhibition of 5 assay repeats of triplicate extraction.

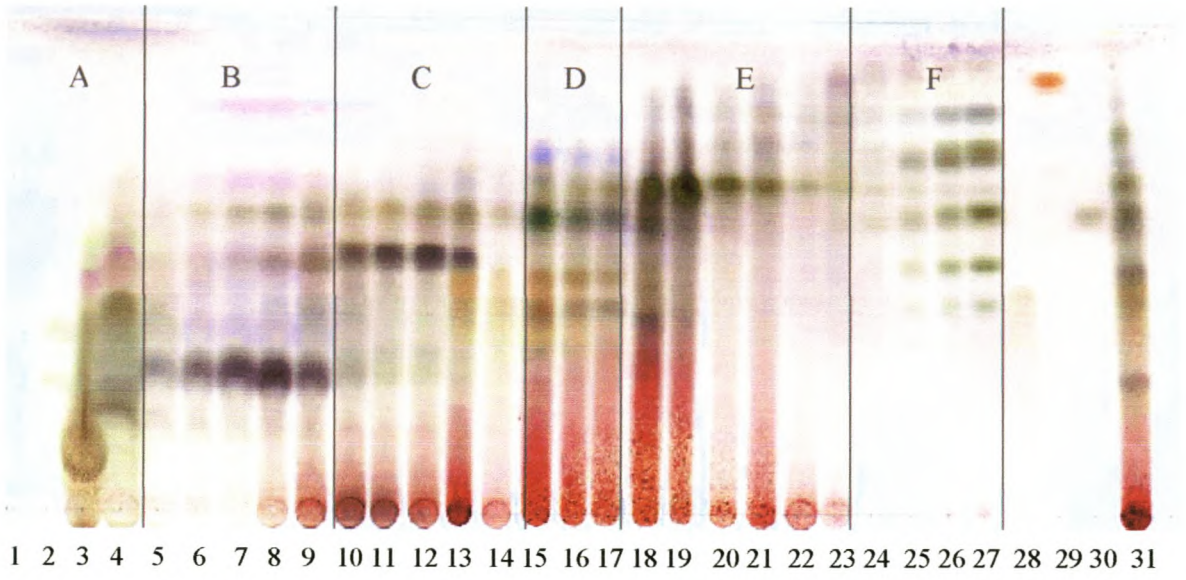

Figure 4. XAD fractions of a methanol extract of unfermented *Cyclopia intermedia* (with prior dichloromethane extraction), spotted (at equal concentration) on silica F_{254} TLC plates and visualised with *p*-anisaldehyde. Lane 1 is the pre-fraction, lanes 2-27 are fractions $f_1 - f_{26}$ and lanes 28, 29, 30 and 31 are mangiferin, hesperetin, hesperidin and the methanol extract, respectively. Fractions within the lines were pooled to form fraction A to F. The methanol extract was fractionated on XAD-1180 polymeric beads by elution with 15%, 30%, 50%, 80% and 100% methanol:water.

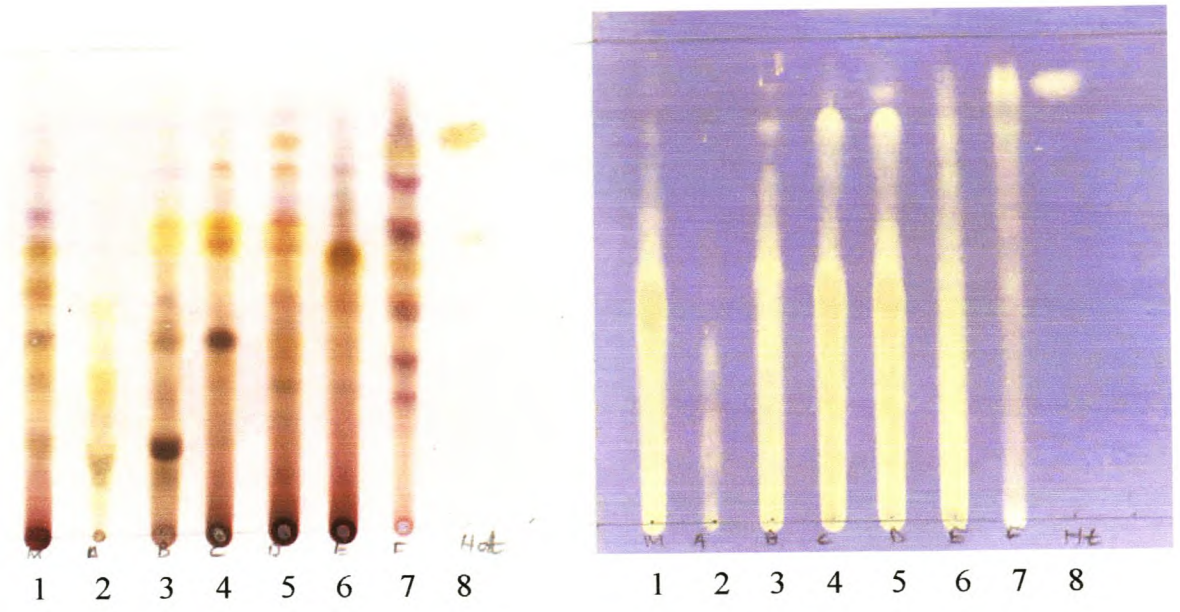

Figure 5. Pooled XAD fractions (A-F) of a methanol extract if unfermented *Cyclopia intermedia* (with prior dichloromethane extraction) spotted (at equal concentration) on silica F_{254} TLC plates and visualised with *p*-anisaldehyde (left) and DPPH$^{\bullet}$ (right). Spots with hydrogen donating abilities cause the DPPH$^{\bullet}$ spray reagent to change from purple to yellow. Lane 1 is the methanol extract, lanes 1-7 are fraction A-F and lane 8 is hesperetin. Fractionation carried out according to the legend of Figure 3.

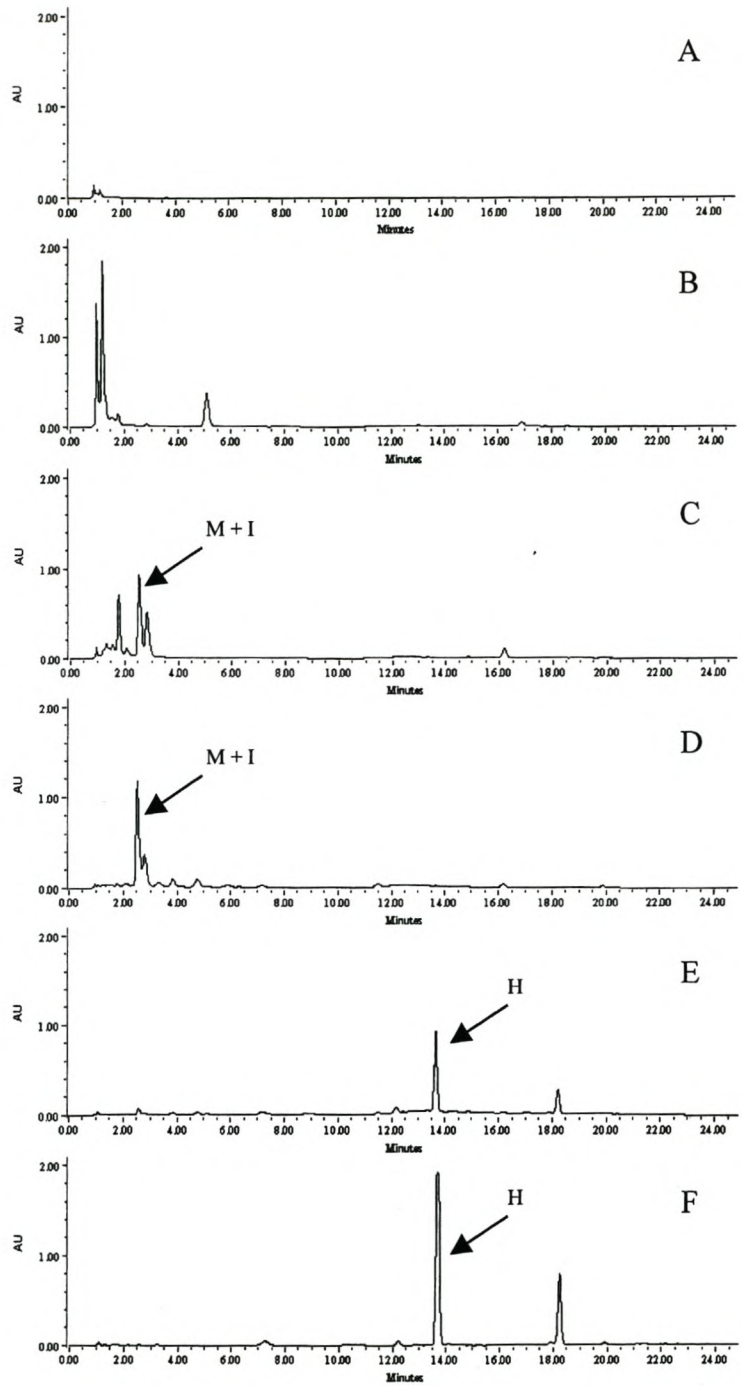

Figure 6. Chromatograms of XAD fractions (A – F) of a methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) with major compounds mangiferin + isomangiferin (M + I) and hesperidin (H). HPLC analysis was carried out on a Zorbax SB-C_{18} (3.5 µm particle size, 150 x 3 mm internal diameter) column under gradient conditions; mobile phase, acetic acid:acetonitrile; flow rate, 1 $ml.min^{-1}$; detection, 280 nm; column temperature, 30°C. Fractionation as described in the legend of Figure 3.

quantified. TLC separation showed several spots with different Rf values in all fractions (Figure 4). The major compounds present were mangiferin + isomangiferin in fraction D and hesperidin in fraction E and F (Figure 5). The concentrations of the compounds, present in the fractions at quantifiable levels, are given in Table 6. Hesperidin made up 40% of fraction F, while mangiferin + isomangiferin made up 15% of fraction D. Fraction A did not contain significant quantities of phenolic compounds, while fraction B had small quantities of mangiferin + isomangiferin, hesperidin and eriodictyol. Luteolin was only present in quantifiable amounts in fraction D while small amounts of eriodictyol could be detected in all fractions except fraction A (Table 6).

The apparent contribution that these compounds made to the total polyphenol content as gallic acid equivalents is given in Table 7. For fractions C and D the apparent contribution made by mangiferin + isomangiferin was 17 and 20%, respectively. Hesperidin accounted for 10% of the total polyphenol content of fraction E, while, it accounted for 78% of the total polyphenol content of fraction F. In total 81% of the total phenolic compounds in fraction F could be accounted for by its mangiferin + isomangiferin, hesperidin and eriodictyol contents. For fractions C, D and E between 14 and 22% of their total polyphenol contents could be accounted for by the phenolic compounds quantified by HPLC.

Antioxidant and antimutagenic activities of the XAD fractions (A-F)

The relative $ABTS^{\bullet+}$ scavenging and ferric reducing activities of the fractions A-F (Table 5) could be ranked in the same order (D > C > E > B > F > A). A very good correlation between these two assays was obtained for the fractions. Fraction D gave the highest antioxidant activity of 2462 and 5103 μmol g^{-1} for the $ABTS^{\bullet+}$ and FRAP assays, respectively. The respective activities of fraction C was slightly less at 2438 and 4758 μmol g^{-1}. Fraction E exhibited 50% of the activity of the most active fractions, D and C. The activities of fractions A, B and F were respectively 6, 30 and 20% of that of fraction D. The antioxidant activities of the fractions were highly correlated with their total polyphenol content ($r = 0.96$; $P < 0.05$). The difference in polyphenol potency of the fractions did not differ as much, although differences were still apparent (Table 5). As for extract activity fractions C and D had the highest polyphenol potency in both the $ABTS^{\bullet+}$ and FRAP assays. The polyphenol potency of the fractions could be ranked as follows: C > D > A > E > F > B ($ABTS^{\bullet+}$ assay) and D > C > E > F > B > A (FRAP assay). Assuming no synergistic or antagonistic effects the contribution by the major compounds, i.e. mangiferin +

isomangiferin and hesperidin, were responsible for the greatest contribution to the $ABTS^{\bullet+}$ scavenging and ferric reducing activities of fractions A-F (Table 8). However their contribution to $ABTS^{\bullet+}$ scavenging activity was less than for ferric reducing activity. Hesperidin was substantially less active in the $ABTS^{\bullet+}$ assay, contributing 30% of the $ABTS^{\bullet+}$ scavenging activity of fraction F, opposed to 64% of the ferric reducing ability (Table 8).

The ability of the fractions to inhibit lipid peroxidation did not follow the same trend as for their $ABTS^{\bullet+}$ scavenging and ferric reducing activities, nor did it correlate with total polyphenol content ($r = 0.06$; $P > 0.05$) (Table 5). The fractions could be ranked as follow: D > F > E > C > B > A. Fraction D was only slightly better than fractions F and E, whereas in the $ABTS^{\bullet+}$ and FRAP assays, fraction F and E had only 25 and 50% of the activity of fraction D, respectively. Fraction A with poor $ABTS^{\bullet+}$ scavenging and ferric reducing activities, also performed poorly against lipid peroxidation in rat liver microsomes and was much less effective than fractions B-F.

The antimutagenic activity of fractions A-F showed that the most non-polar fractions were the most effective in reducing the number of revertants (Table 5). Fractions A-C exhibited no protective effects against 2-AAF induced mutagenesis, while fractions D-F exhibited moderate to good antimutagenic activity. Fraction F inhibited mutagenesis the most (85%) followed by fraction E (77%). Depending on concentration, fractions A-E displayed antimutagenic or promutagenic activity (Figure 6). At low concentrations (1 mg $plate^{-1}$) fractions A-E had promutagenic activity, while at a concentration of 2 mg $plate^{-1}$ only fractions A, C and D still exhibited promutagenic activity. At 4 and 8 mg $plate^{-1}$ only fraction A was promutagenic, although to a lesser extent than at the lower concentrations. Fractions with promutagenic effects were not mutagenic towards TA98 in the presence and absence of S9 (Table 9).

Analysis of polyphenol content and major compounds of C_{18} fractions (E_1-E_6)

The fractions f_1-f_{139}, spotted on silica thin layer plates and visualised with *p*-anisaldehyde, were pooled as depicted in Figures 8 and 9. The pooled fractions and their response to the $DPPH^{\bullet}$ spray reagent (Figure 10) shows that all fractions exhibited hydrogen donating abilities and thus antioxidant activity. Fraction E_1 represented 25% of fraction E (after filtration) applied to the column with the remaining fractions representing between 8-14% (Table 10). The total polyphenol content of fractions E_1-E_6 decreased with decreasing

Table 5. Yield, total polyphenol content (TP), radical scavenging (TAA) and ferric reducing abilities (FRAP), ability to inhibit Fe^{2+} induced rat liver microsomal lipid peroxidation (TBARS) and antimutagenic activity (Ames) of XAD fractions (A-F)[a] of a methanol extract (with prior dichloromethane extraction) of unfermented *C. intermedia.*

Fraction	Yield[b]	TP[c]	TAA[d]	FRAP[e]	TBARS[f]	Ames[g]
A	24.63	2.94 (0.72)	148.0 (5034)	184.4 (6272)	14.33	416.6 (-10.37)
B	6.25	21.62 (1.35)	751.7 (3476)	1699 (7858)	46.56	319.8 (15.29)
C	21.6	37.52 (8.10)	2438 (6497)	4758 (12681)	48.89	353.9 (6.23)
D	11.98	39.82 (4.77)	2462 (6182)	5103 (12815)	56.71	276.9 (26.66)
E	18.50	28.45 (5.26)	1323 (4650)	2919 (10260	54.86	94.94 (74.85)
F	2.85	13.07 (0.37)	516.8 (3954)	1075 (8224)	55.28	59.42 (84.26)

[a] Values are the means of 3 replicates (TP, ABTS, FRAP and TBARS) or 5 replicates (Ames). Fractionation as described in the legend of Figure 3.

[b] Expressed as g extract 100 g^{-1} methanol extract.

[c] Expressed as g gallic acid equivalents 100 g^{-1} fraction and as g gallic acid equivalents 100 g^{-1} methanol extract in brackets.

[d] Expressed as μmol Trolox equivalents g^{-1} fraction and μmol Trolox equivalents g^{-1} gallic acid equivalents in brackets.

[e] Expressed as μmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} fraction and μmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} gallic acid equivalents in brackets.

[f] Percentage inhibition measured as thiobarbituric reactive substances (TBARS) on an equal dry fraction mass basis.

[g] Expressed as revertants $plate^{-1}$ against the metabolically activated mutagen 2-acetylaminofluorene (2-AAF) (5 μg $plate^{-1}$) in TA98. The mean number of 2-AAF induced revertants $plate^{-1}$ was 377.5 ± 53.38 and the frequency of spontaneous reversion was 29.46 ± 5.32. The fraction concentration was 4 mg $plate^{-1}$. Percentage inhibition is given in brackets.

Table 6. Concentration[a] of the major phenolic compounds of XAD fractions (A – F)[b] of a methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction).

Fraction	M + I[c]	Hd[d]	E[e]	L[f]	N[g]	Ht[h]	F[i]
A							
B	0.45	0.05	0.03				
C	11.65	0.06	0.72				
D	14.83	0.09	0.35	0.33			
E	2.29	10.43	0.17				
F	0.37	39.13	0.14				

[a] HPLC analysis was done in duplicate on a Zorbax SB-C_{18} (3.5 μm particle size, 150 x 3 mm) column under gradient conditions: mobile phase, acetic acid:acetonitrile; flow rate, 1 ml min^{-1}; detection, 280 nm; column temperature, 30°C. Concentration of compounds is expressed as g 100 g^{-1} fraction. Concentrations of compounds omitted from the table were present in only small quantities that were outside the detection limits of the procedure used.

[b] Fractionation as described in the legend of Figure 3.

[c] Mangiferin + isomangiferin (M + I) was quantified using mangiferin as standard due to their co-elution. Isomangiferin was assumed to give the same absorbance as mangiferin.

[d] Hesperidin (Hd).

[e] Eriodictyol (E).

[f] Luteolin (L).

[g] Naringenin (N).

[h] Hesperetin (Ht).

[i] Formononetin (F).

Table 7. Apparent contribution of the major phenolic compounds of XAD fractions (A–F)[a] of a methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) to their total polyphenol content as gallic acid equivalents.

	Fraction[c]					
Compound[b]	**A**	**B**	**C**	**D**	**E**	**F**
	2.94	21.62	37.52	39.82	28.45	13.07
Mangiferin + Isomangiferin y = 6.2571x + 0.012 (r = 0.999)		0.25	6.37	8.10	1.25	0.20
Hesperidin y = 2.9777x + 0.004 (r = 0.998)		0.01	0.02	0.02	2.72	10.19
Eriodictyol y = 11.595x + 0.102 (r = 0.978)		0.03	0.73	0.36	0.17	0.14
Luteolin y = 10.716x + 0.095 (r = 0.999)				0.31		

[a] Fractionation as described in the legend of Figure 3.

[b] The equation for the line (where y = absorbance and x = mg mL^{-1} compound in the reaction mixture) was calculated from a dilution series consisting of 5 points diluted to give an absorbance range of 0.2-0.8.

[c] Gallic acid equivalents of the fractions calculated as g 100 g^{-1} fraction [the equation for gallic acid was y = 11.42x + 0.0245 (r = 0.999)].

Table 8. Apparent contribution of the major phenolic compounds of XAD fractions (A–F) of a methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) to radical scavenging (TAA) and ferric reducing activities (FRAP).

Compound[b]	TAA[c]						Compound[d]	FRAP[e]					
	Fraction[f]							Fraction[f]					
	A	B	C	D	E	F		A	B	C	D	E	F
	148.0	751.7	2438	2462	1323	516.8		184.4	1699	4758	5103	2919	1075
Mangiferin + Isomangiferin y = 6912.9x + 0.7843 (r = 0.988)		17.24	446.3	568.1	87.73	14.17	Mangiferin + Isomangiferin y = 0.0746x + 0.0204 (r = 0.999)		42.34	1059	1351	209.8	35.05
Hesperidin y = 1033.1x + 8.4367 (r = 0.899)		0.19	0.24	0.36	41.30	154.9	Hesperidin y = 0.0208x + 0.0948 (r = 0.998)		6.07	6.25	6.77	188.3	692.3
Eriodictyol y = 3397.1x + 5.1345 (r = 0.986)		0.83	19.86	9.65	4.69	3.86	Eriodictyol y = 0.0751x + 0.0607 (r = 0.988)		1.21	93.87	44.18	20.01	15.98
Luteolin y = 4160.3x + 8597 (r = 0.999)				11.22			Luteolin y = 0.0316x + 0.0063 (r = 0.999)				19.41		

[a] Fractionation as described in the legend of Figure 3.

[b] The equation for the line (where y = % inhibition and x = µmol compound in the reaction mixture) was calculated from a dilution series consisting of 5 points with a inhibition of 20-80%.

[c] Radical scavenging activity determined with the $ABTS^{\bullet+}$ assay (TAA) expressed as µmol Trolox equivalents g^{-1} dry fraction.

[d] The equation for the line (where y = absorbance at 593 nm and x = µmol compound in the reaction mixture) was calculated from a dilution series consisting of 5 points with an absorbance of 0.2-0.8.

[e] Ferric reducing activity determined with the FRAP assay expressed as µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} dry fraction.

[f] Trolox and $FeSO_4 \bullet 7H_2O$ equivalents of the fractions calculated with the equations y = 4273x + 0.1308 (r = 0.999) and y = 0.0194x + 0.006 (r = 0.999), respectively.

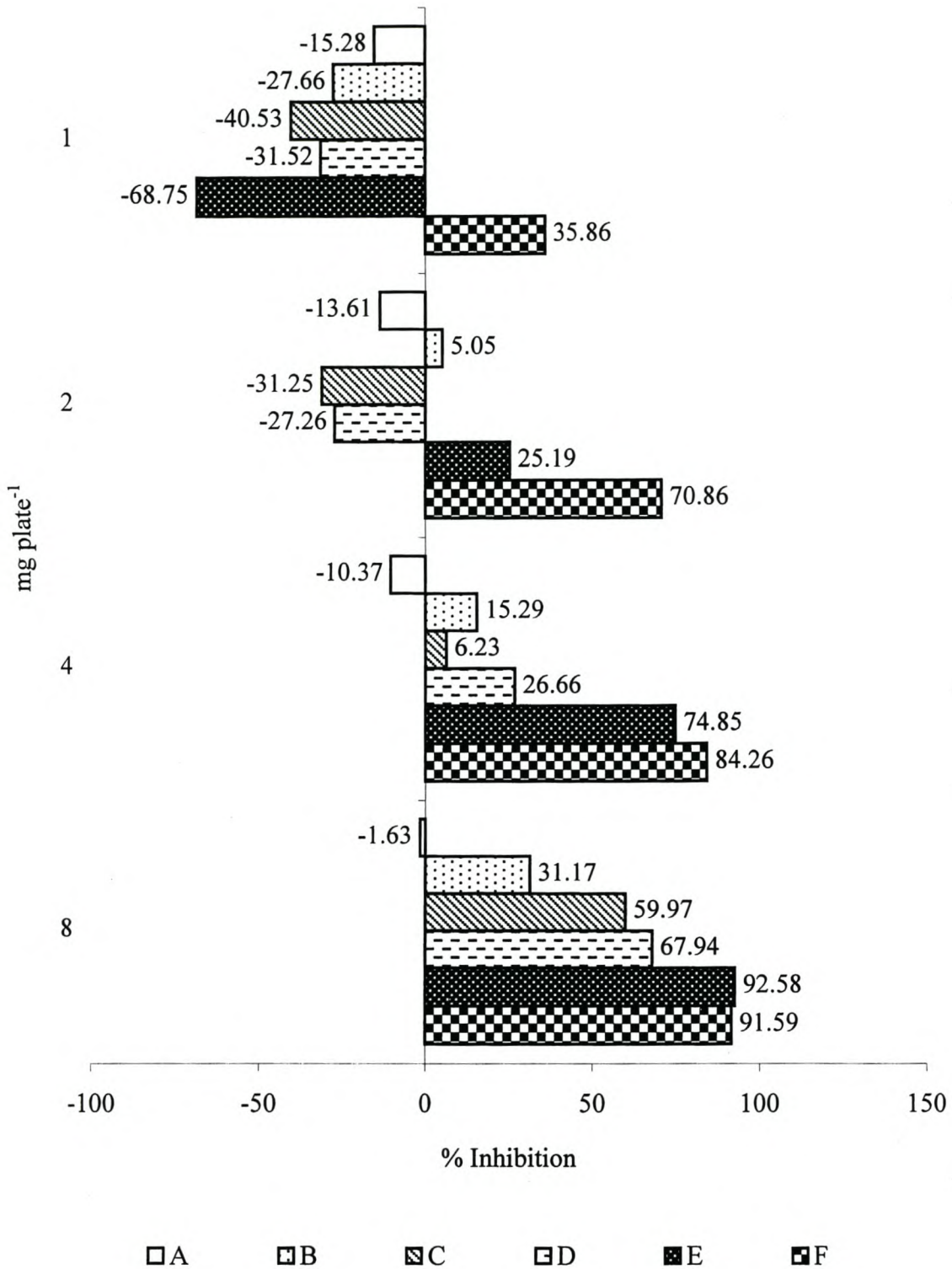

Figure 7. Dose response antimutagenic and promutagenic activities of XAD fractions (A-F) from a methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) against 2-acetylaminofluorene in the *S. typhimurium* plate-incorporation assay. Values are the mean ± S.D. % inhibition of 5 plates per assay. Fractionation carried out as described in the legend of Figure 3.

Table 9. Effect of XAD[a] fractions on the number of revertants in TA98 with and without metabolic activation in the *S. typhimurium* plate incorporation assay .

	Revertants[b]							
	1 mg plate^{-1}		2 mg plate^{-1}		4 mg plate^{-1}		8 mg plate^{-1}	
	+S9	*-S9*	*+S9*	*-S9*	*+S9*	*-S9*	*+S9*	*-S9*
A	47	39	47	43	51	31	53	50
B	42	43						
C	36	32	43	32				
D	36	30	41	33				
- Control[c]	34.4 ± 5.03							
+ Control[d]	480.8 ± 11.54							

[a] Fractions A, B, C and D. Fractionation carried out according to the legend of Figure 3.

[b] Number of revertants with and without the addition of S9 using tester strain TA98 of fractions exhibiting promutagenic effects. Each value represents the mean of analysis done in triplicate except for the negative and positive controls that had 5 replicates.

[c] Number of spontaneous revertants ± S.D. in TA98.

[d] Number of revertants ± S.D. induced by 2-acetylaminofluorene (5 μg plate^{-1}) in TA98 with metabolic activation.

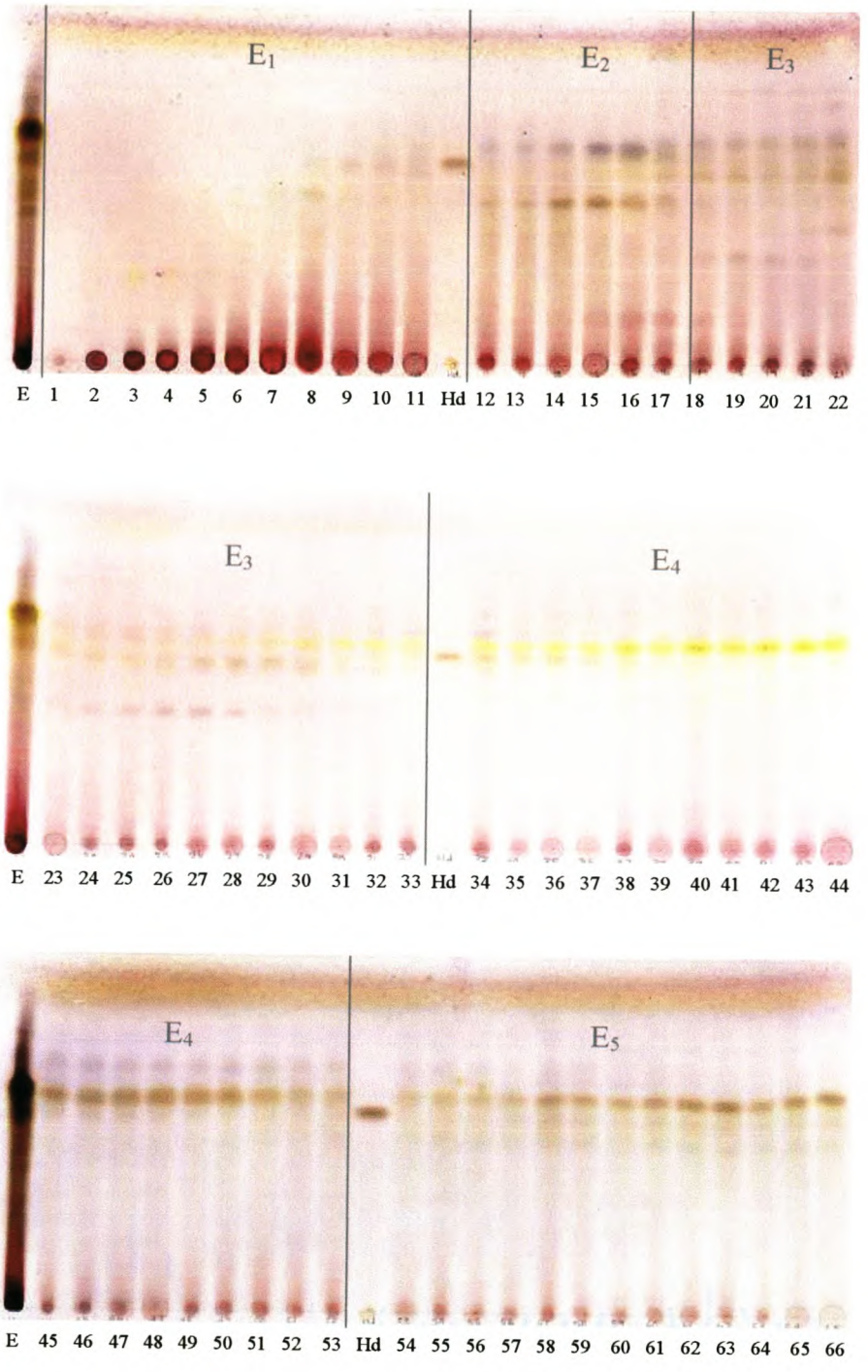

Figure 8. C_{18} fractions from fraction E of the fractionated methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) spotted on silica F_{254} thin layer plates and visualised with *p*-anisaldehyde. Lane 1 is the pre-fraction and 2-67 is f_1-f_{66}, lane E is fraction E (filtered) and lane Hd is hesperidin. Fractionation of fraction E was done on reversed-phase Bondesil-C_{18} (40 µm) with a 50%-100% methanol:water elution gradient. Fractions within the lines were pooled to form fraction E_1 to E_6.

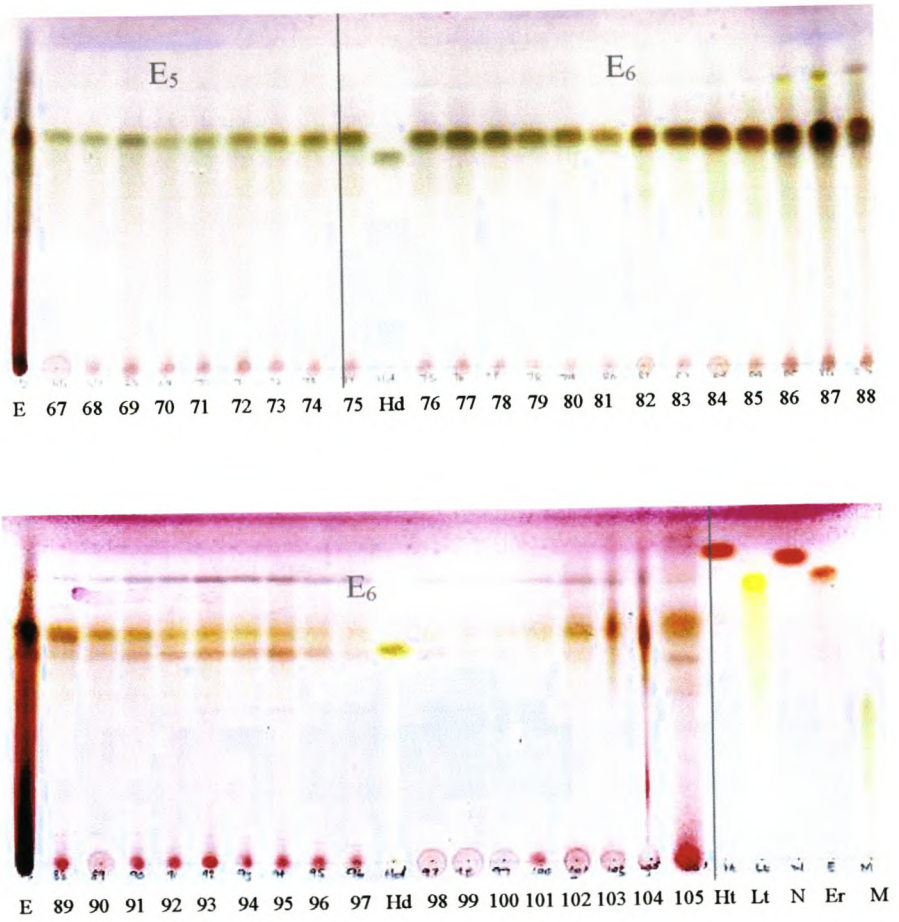

Figure 9. C_{18} fractions from fraction E of the fractionated methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) spotted on silica F_{254} thin layer plates and visualised with *p*-anisaldehyde. Lane 68-105 are f_{67}-f_{104} and lane 107 is the pooled fractions f_{105} to f_{139}, lane E was fraction E (filtered), lane Ht, Lt, N, Er and M were hersperetinm luteolin, naringenin, eriodictyol and mangiferin, respectively. Fractionation as described in the legend of Figure 8.

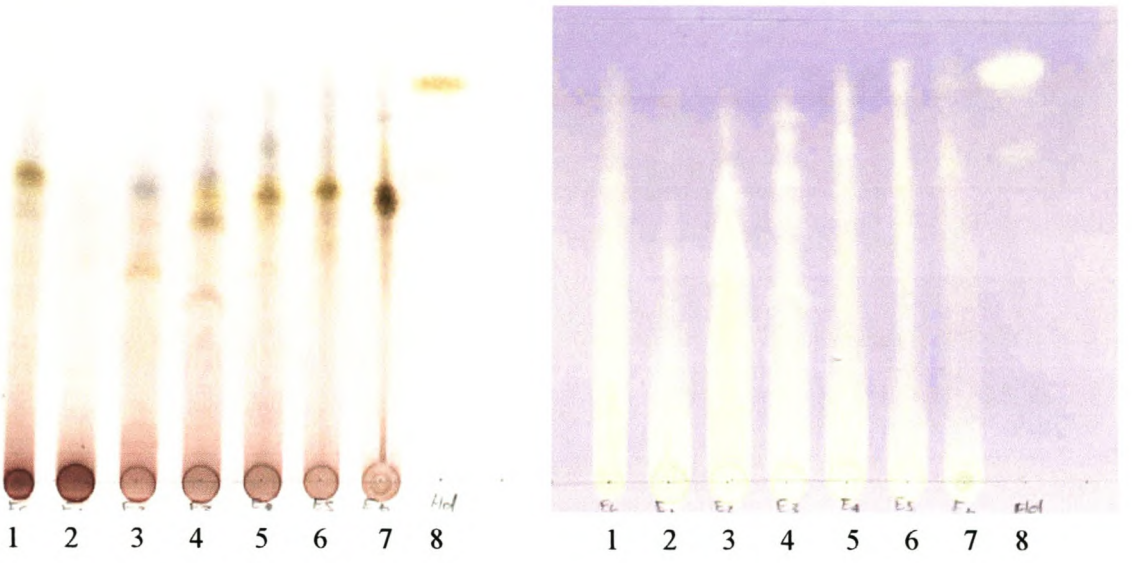

Figure 10. Pooled C_{18} fractions (E_1-E_6) from fraction E of the fractionated methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) spotted (at equal concentrations) on silica F_{254} thin layer plates and visualised with *p*-anisaldehyde (left) and $DPPH^{\bullet}$ (right). Spots with hydrogen donating abilities cause the $DPPH^{\bullet}$ spray reagent to change from purple to yellow. Lane 1 is fraction E (filtered), lanes 2-7 are fraction E_1-E_6 and lane 8 is hesperetin. Fractionation as described in the legend of Figure 7.

polarity of the eluting solvent (Table 10). The total polyphenol content was highest for fraction E_1 and E_2 (*c.* 36%), however E_1 constituting 9% of the polyphenols of fraction E while E_2 only constituted *c.* 4.4% of the polyphenols of fraction E.

HPLC analyses of fractions E_1-E_6 (Figure 11) identified mangiferin + isomangiferin in fraction E_2 and hesperidin in fractions E_3 and E_4 as the major compounds. There were also several unidentified peaks present in fraction E_2, and in fraction E_5. A distinct unidentified peak present in fraction E_5 was also present in the XAD fraction F (Figure 5). Fraction E_2 contained 8% mangiferin + isomangiferin, while fraction E_3 and E_2 contained 1 and 3% hesperidin, respectively (Table 11). The small quantities of luteolin, naringenin and hesperetin present in fraction E_6 ranged from 1% for luteolin to 0.1 and 0.06% for naringenin and hesperetin, respectively. The insoluble residue (R) filtered out of fraction E before fractionation contained appreciable amounts of hesperidin (15%) (Table 11). The contribution that these phenolic compounds made to the total polyphenol content (Table 12) ranged from between 0.1% and 18%. The highest contributions were made by mangiferin + isomangiferin for fraction E_2 (12%) and hesperidin for fraction E_4 (3%). Whilst the total polyphenol content of the insoluble residue was 23%, 17% was attributable to hesperidin. For fractions E_4, E_5 and E_6 the polyphenolic compounds identified only made a small contribution to the total polyphenol content (Table 12).

Antioxidant and antimutagenic activity of C_{18} fractions (E_1-E_6)

The $ABTS^{\bullet+}$ scavenging and ferric reducing activities of the C_{18} fractions decreased with decreases in total polyphenol content (Table 10). A very good correlation ($r \geq 0.98$; $P < 0.05$) was obtained for both activities with total polyphenol content. Fraction E_1 accounted for 37 and 39% of the $ABTS^{\bullet+}$ scavenging and ferric reducing abilities of fraction E (filtered), respectively, while fractions E_2-E_6 accounted for 15, 13, 10, 6 and 5% of the $ABTS^{\bullet+}$ scavenging and 7, 15, 12, 6 and 5% of the ferric reducing activity, respectively. The $ABTS^{\bullet+}$ scavenging and ferric reducing activity of the polyphenols (i.e. their potency) was the highest for fraction E_1 and slightly less for the remaining fractions (Table 10). The ferric reducing activity of the residue (R) relative to fraction E (filtered) was much higher than for $ABTS^{\bullet+}$ scavenging activity.

Assuming no synergistic effects, the contribution that the quantified compounds (Table 13) made to the $ABTS^{\bullet+}$ scavenging and ferric reducing activities of the fractions was

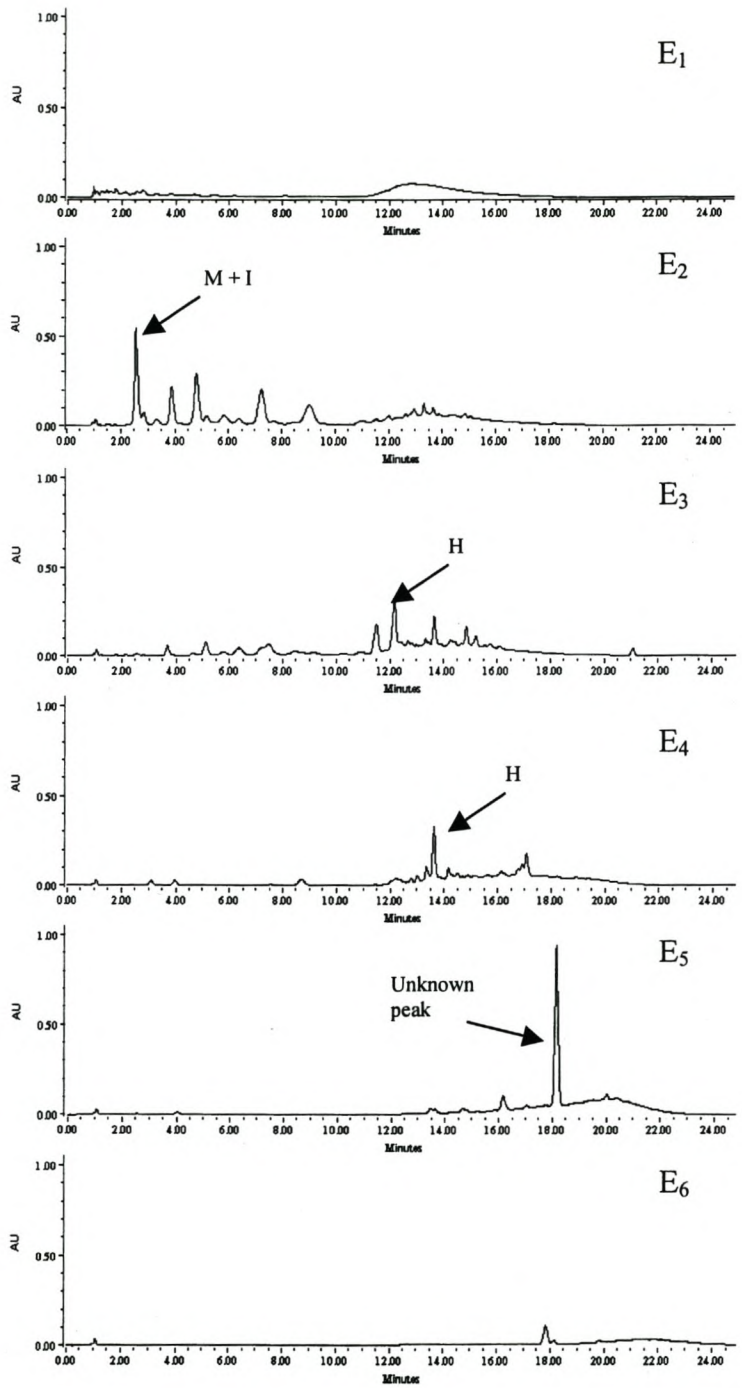

Figure 11. Chromatograms of C_{18} fractions ($E_1 - E_6$) from the XAD fraction E with major compounds mangiferin + isomangiferin (M + I) and hesperidin (H). HPLC analysis was carried out on a Zorbax SB-C_{18} (3.5 µm particle size, 150 x 3 mm) column under gradient conditions; mobile phase, acetic acid:acetonitrile; flow rate, 1 $ml.min^{-1}$; detection, 280 nm; column temperature, 30°C. Fractionation carried out according to the legend of Figure 7.

greatest for fraction E_2 (12 and 18% of the respective activity). This contribution to the activity was mostly due to its mangiferin + isomangiferin content. The hesperidin in fraction E_4 was accountable for only 0.7% of the $ABTS^{\bullet+}$ scavenging activity and 2% of its ferric reducing activity. The luteolin present in fraction E_6 contributed 5.7 and 6.3% of its $ABTS^{\bullet+}$ scavenging and ferric reducing activities, respectively. Hesperidin had an apparent contribution of between 13 and 14% of the $ABTS^{\bullet+}$ and ferric reducing activities of the residue (R).

The ability of the fractions to inhibit lipid peroxidation did not differ greatly from fraction to fraction (Table 10). Activity was within the range 67-74% for all of the fractions. No significant correlation with the total polyphenol content was obtained ($r = 0.257$; $P > 0.05$). The residue (R), consisting largely of hesperidin (Table 11), had the weakest ability to inhibit lipid peroxidation (54%) at the concentration tested.

Unlike the ability to inhibit lipid peroxidation the antimutagenic activity of the fractions against 2-AAF induced mutagenesis differed considerably (Table 10). The highest antimutagenic activity was exhibited by fraction E_6 (85% inhibition), followed by fraction E_1 (74% inhibition). Fractions E_3 and E_4 with similar antioxidant activity in all the assays, had similar antimutagenic activity of 57 and 61%, respectively. This was not the same for fractions E_2 and E_5 that displayed similar antimutagenic activity (44 and 42%, respectively), but showed considerable differences in $ABTS^{\bullet+}$ scavenging and ferric reducing activities (Table 10). The residue (R) had a promutagenic effect (94%). The activity of the fractions was concentration dependent (Figure 12). At the lowest concentration tested (1 mg $plate^{-1}$) fractions E_1, E_2 and E_4 were antimutagenic, as opposed to fractions E_3, E_5 and E_6 that were promutagenic. However, these fractions did not show mutagenic activity in the presence and absence of S9 (Table 14), nor were they mutagenic at 2 and 4 mg $plate^{-1}$. Similarly the residue (R), that was promutagenic at 1 and 2 mg $plate^{-1}$, was not mutagenic in TA98 in the presence and absence of metabolic activation (Table 14).

DISCUSSION

The antioxidant (Bors *et al.*, 1990; Mora *et al.*, 1990; Sichel *et al.*, 1991; Rice-Evans *et al.*, 1996; Rice-Evans & Miller, 1998) and antimutagenic (Choi *et al.*, 1994; Edenharder & Tang, 1997; Nakasugi & Komai, 1998; Nakasugi *et al.*, 2000) activities of phenolic compounds

Table 10. Yield, total polyphenol content (TP), radical scavenging (TAA) and ferric reducing abilities (FRAP), ability to inhibit Fe^{2+} induced rat liver microsomal lipid peroxidation (TBARS) and antimutagenic activity (Ames) of C_{18} fractions (E_1-E_6)[a] from the fraction E of the fractionated methanol extract (XAD column) of unfermented *C. intermedia* (with prior dichloromethane extraction).

Fraction	Yield[b]	TP[c]	TAA[d]	FRAP[e]	TBARS[f]	Ames[g]
E[h]		24.88	1806 (7258)	2675 (10751)	83.48	143 (64.11)
R[h]		23.00	469.1 (2039)	1928 (8382)	53.94	771.4 (-93.63)
E_1	25.18	36.34 (9.15)	2650 (7292)	4151 (11422)	72.86	102.3 (74.33)
E_2	11.07	35.96 (3.98)	2480 (6896)	4002 (11129)	69.85	221.3 (44.46)
E_3	14.43	27.95 (4.03)	1597 (5713)	2790 (9982)	71.03	170.2 (57.27)
E_4	12.58	23.59 (2.96)	1485 (6295)	2469 (10466)	67.14	154.5 (61.21)
E_5	8.79	19.68 (1.73)	1207 (6133)	1845 (9375)	73.18	230.4 (42.16)
E_6	13.48	10.92 (1.47)	689.5 (6314)	1044 (9560)	73.27	59.00 (85.19)

[a] Values are the means of analysis done with three replicates (TP, ABTS, FRAP and TBARS) and 5 replicates (Ames). Fraction as described in the legend of Figure 7.

[b] Expressed as g extract 100 g^{-1} filtered fraction E.

[c] Expressed as g gallic acid equivalents 100 g^{-1} fraction and g gallic acid equivalents.100 g^{-1} fraction E (filtered) in brackets.

[d] Expressed as µmol Trolox equivalents g^{-1} fraction and µmol Trolox equivalents g^{-1} gallic acid equivalents in brackets.

[e] Expressed as µmol $Fe(II)SO_4 \bullet 7H_2O$ equivalents g^{-1} fraction and µmol $Fe(II)SO_4 \bullet 7H_2O$ equivalents g^{-1} gallic acid equivalents in brackets.

[f] Percentage inhibition measured as thiobarbituric reactive substances (TBARS) on an equal fraction mass basis for fractions E_1 – E_6 while % inhibition of fraction E and R are on an equal mass basis with that from Table 5 (fractions A – F).

[g] Expressed as revertants $plate^{-1}$ against the metabolically activated mutagen 2-acetylaminofluorene (2-AAF) (5 µg $plate^{-1}$) in TA98. The mean number of 2-AAF induced revertants $plate^{-1}$ was 398.41 ± 71.88 and the frequency of spontaneous reversion was 21.58 ± 7.19. The fraction concentration was 4 mg $plate^{-1}$. Percentage inhibition given in brackets.

[h] Fraction E obtained from the fractionation procedure as described in the legend of figure 3 was filtered and fractionated further on C_{18}. The filtrate (E) and the residue (R) were tested for activity.

Table 11. Concentration[a] of the major phenolic compounds of C_{18} fractions ($E_1 - E_6$)[b] from fraction E of the fractionated methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction).

Fraction	M + I[c]	Hd[d]	E[e]	L[f]	N[g]	Ht[h]	F[i]
R[j]	0.08	15.13	0.25				
E_1	0.095						
E_2	7.97	0.04					
E_3	0.09	1.23	0.07				
E_4	0.03	2.68	0.21				
E_5	0.23						
E_6	0.17			1.15	0.11	0.06	

[a] HPLC analysis was done in duplicate on a Zorbax SB-C_{18} (3.5 µm particle size, 150 x 3 mm) column under gradient conditions: mobile phase, acetic acid:acetonitrile; flow rate, 1 ml min^{-1}; detection, 280 nm; column temperature, 30°C. Concentration of compounds is expressed as g.100 g^{-1} fraction. Concentrations of compounds omitted from the table were present in only small quantities that were outside the detection limits of the procedure used.

[b] Fractionation as described in the legend of figure 7.

[c] Mangiferin + isomangiferin (M + I) were quantified using mangiferin as standard due to their co-elution. Isomangiferin was assumed to give the same absorbance as mangiferin.

[d] Hesperidin (Hd).

[e] Eriodictyol (E).

[f] Luteolin (L).

[g] Naringenin (N).

[h] Hesperetin (Ht).

[i] Formononetin (F)

[j] Fraction E was suspended in 50% methanol:water and filtered before being fractionated. The phenolic compounds in the residue (R) were quantified as for fractions $E_1 - E_6$.

Table 12. Apparent contribution of the major phenolic compounds of C_{18} fractions (E_1–E_6)[a] from fraction E of the methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) to their total polyphenol content expressed as gallic acid equivalents.

	Fraction[c]						
Compound[b]	**E_1**	**E_2**	**E_3**	**E_4**	**E_5**	**E_6**	**R[d]**
	36.34	35.96	27.95	23.59	19.68	10.92	23.00
Mangiferin + Isomangiferin y = 6.2571x + 0.012 (r = 0.999)	0.05	4.43	0.05	0.16	0.13	0.09	0.04
Hesperidin y = 2.9777x + 0.004 (r = 0.998)		0.01	0.32	0.69			3.94
Eriodictyol y = 11.595x + 0.102 (r = 0.978)			0.07	0.21			0.26
Luteolin y = 10.716x + 0.095 (r = 0.999)						1.08	
Naringenin y = 9.3026x + 0.024 (r = 0.999)						0.09	
Hesperetin y = 8.8034x + 0.051 (R^2 = 0.9974)						0.05	

[a] Fractionation as described in the legend of Figure 7.

[b] The equation for the line was calculated from a dilution series (5 concentrations) diluted to give an absorbance range of between 0.2 and 0.8.

[c] Gallic acid equivalents of the fractions calculated as g.100 g^{-1} fraction [the equation for gallic acid was y = 11.42x + 0.0245 (r = 0.999)].

[d] Fraction E obtained from the fractionation procedure as described in the legend of figure 3 was filtered to yield a redsidue (R) and the filtrate that was fractionated further on C_{18} to yield fractions E_1-E_6.

Table 13. Antioxidant activities of the major phenolic compounds the of C_{18} fractions (E_1–E_6)[a] from fraction E of the methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) in the $ABTS^{\bullet+}$ scavenging (TAA) and ferric reducing (FRAP) assays.

Compound[b]	TAA[c]							Compound[d]	FRAP[e]						
	Fraction[f]								Fraction[f]						
	E_1[g]	E_2	E_3	E_4	E_5	E_6	R[g]		E_1[g]	E_2	E_3	E_4	E_5	E_6	R[g]
	2650	2480	1597	1485	1207	689.5	469.1		4151	4002	2790	2469	1845	1044	1928
Mangiferin + Isomangiferin y = 6912.9x + 0.7843 (r = 0.988)	3.64	305.3	3.45	1.15	8.81	6.51	3.06	Mangiferin + Isomangiferin y = 0.0746x + 0.0204 (r = 0.999)	10.01	727.1	9.56	4.09	22.30	2.91	8.65
Hesperidin y = 1033.1x + 8.4367 (r = 0.899)		0.16	4.87	10.61			59.91	Hesperidin y = 0.0208x + 0.0948 (r = 0.998)		5.89	26.79	52.26			270.9
Eriodictyol y = 3397.1x + 5.1345 (r = 0.986)			1.93	5.79			6.89	Eriodictyol y = 0.0751x + 0.0607 (r = 0.988)			6.58	25.38			30.75
Luteolin y = 4160.3x + 8597 (r = 0.999)						39.12		Luteolin y = 0.0316x + 0.0063 (r = 0.999)						66.08	
Naringenin y = 178.23x + 1.1959 (r = 0.991)						0.17		Naringenin y = 0.0002x + 0.004 (r = 0.998)						0.58	
Hesperetin y = 887.12x + 11.625 (r = 0.879)						0.41		Hesperetin y = 0.0154x + 0.3327 (r = 0.996)						19.03	

[a] Fractionation as described in the footnotes of Figure 7.

[b] The equation for the line (where y = % inhibition and x = µmol compound in the reaction mixture) was calculated from a dilution series (5 concentrations) diluted to give an absorbance range of between 20 and 80% inhibition.

[c] Radical scavenging activity determined with the $ABTS^{\bullet+}$ assay (TAA) expressed as µmol Trolox equivalents g^{-1} fraction.

[d] The equation for the line (where y = absorbance at 593 nm and x = µmol compound in the reaction mixture) was calculated from a dilution series (5 concentrations) diluted to give an absorbance range of between 0.2 and 0.8.

[e] Ferric reducing activity determined with the FRAP assay expressed as µmol $FeSO_4 \bullet 7H_2O$ equivalents g^{-1} fraction.

[f] Trolox and $FeSO_4 \bullet 7H_2O$ equivalents of the fractions calculated with the equations y = 4273x + 0.1308 (r = 0.999) and y = 0.0194x + 0.006 (r = 0.999), respectively.

[g] Fraction E obtained from the fractionation procedure as described in the legend of figure 3 was filtered to yield a redsidue (R) and the filtrate (E) that was fractionated further on C_{18} to yield fractions E_1-E_6.

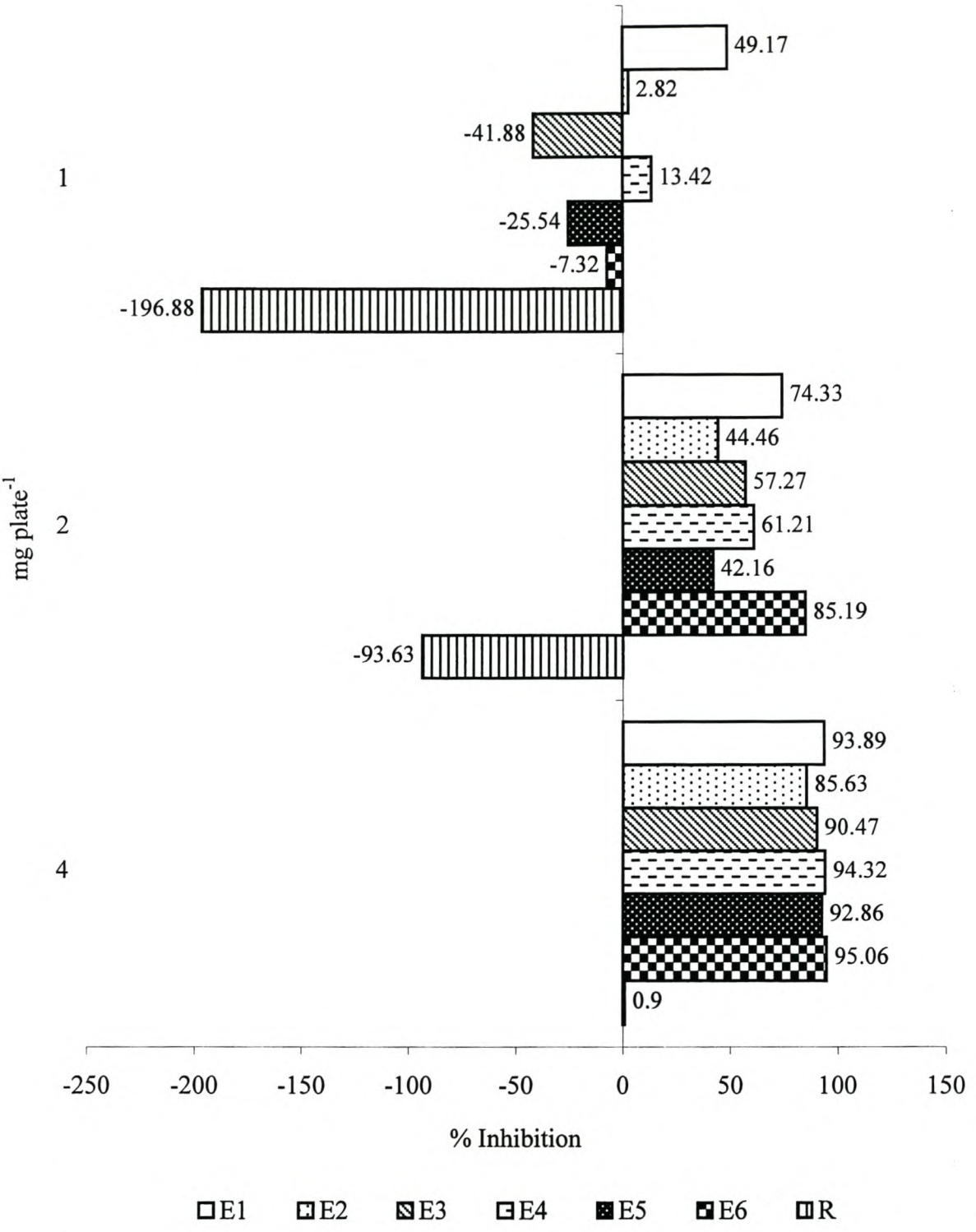

Figure 12. Does response antimutagenic and promutagenic activity of C_{18} fractions (E_1-E_6) from fraction E of the fractionated methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) against 2-acetylaminoflourene in the *S. typhimurium* plate-incorporation assay. Values are the mean ± S.D. % inhibition of 5 assay repeats with triplicate extraction. Fractionation as described in the legend of Figure 8.

Table 14. Effect of C_{18} fractions (E_3-E_6) from fraction E of the methanol extract of unfermented *C. intermedia* (with prior dichloromethane extraction) on the number of revertants in TA98 with and without metabolic activation in the *S. typhimurium* antimutagenicity assay.

	Revertants[b]			
	1 mg plate^{-1}		2 mg plate^{-1}	
	+S9	*-S9*	*+S9*	*-S9*
E_3	39	33		
E_5	43	36		
E_6	35	32		
R[c]	42	32	37	42
- Control[d]	34.4 ± 5.03			
+ Control[e]	480.8 ± 11.54			

[a] Fractions E_3, E_5 and E_6 and the residue (R) filtered out from fraction E. Fractionation as described in the legend of Figure 7.

[b] Number of revertants with and without the addition of S9 in TA98 of fractions exhibiting promutagenic effects. Each value represents the mean of analysis done in triplicate except for the negative and positive controls that had 5 replicates.

[c] Fraction E obtained from the fractionation procedure as described in the legend of figure 3 was filtered to yield a redsidue (R) and the filtrate that was fractionated further on C_{18} to yield fractions E_1-E_6.

[d] Number of spontaenous revertants ± S.D. in TA98.

[e] Number of revertants ± S.D. from mutagenesis induced by 2-acetylaminoflourene (5 μg.plate^{-1}) in TA98 with metabolic activation.

have been extensively studied in various *in vitro* systems in an effort to elucidate their possible structure-activity relationships and/or synergistic, additive or antagonistic effects. In this study the activity of several of the phenolic compounds present in fermented *C. intermedia* were investigated, using various antioxidant assays and an antimutagenic assay. Their reactivity towards the Folin-Ciocalteu reagent was also investigated. The varying degrees of reactivity exhibited by the xanthone mangiferin, the flavanones hesperidin, hesperetin, eriodictyol and naringenin, the flavone luteolin and the isoflavone formononetin towards the Folin-Ciocalteu reagent illustrate that total polyphenol content expressed in terms of gallic acid equivalents may purely be used as a rough guide in the selection process of active extracts or fractions. According to the reactivities obtained for these compounds it must be kept in mind that total polyphenol content may be over- or underestimated, depending on the phenolic composition of the fraction or extract. The presence of formononetin for example would lead to underestimation as it does not react with the Folin-Ciocalteu reagent. Since mangiferin and hesperidin are the major compounds present, expressing total polyphenol content in terms of gallic acid, that is not present at all should be reconsidered. On the other hand, gallic acid is used in most instances which facilitates comparison between plant extracts.

Their relative order of antioxidant activity depended on the assay used. The activity of phenolic compounds as radical scavengers and ferric reducers can be in part explained by their half peak oxidation potentials (Ep/2), said to be a good parameter to describe the scavenging activity of flavonoids (Van Acker *et al.*, 1996). Reactivity of the compounds determined with the FRAP assay were ranked as mangiferin = eriodictyol > luteolin > hesperidin > hesperetin > formononetin > naringenin. The higher activity of luteolin over hesperidin and hesperetin can be explained by its lower Ep/2 of 0.18 V compared to 0.40 and 0.44 V for hesperetin and hesperidin, respectively (Van Acker *et al.*, 1998). Naringenin has a much higher Ep/2 of 0.60 (Van Acker *et al.*, 1998). The lower the Ep/2 value the greater the antioxidant activity of the compound. Compounds with Ep/2 < 0.2 V are considered to be good antioxidants, while those with Ep/2 > 0.2 V as moderate antioxidants (Van Acker *et al.*, 1996). Apart from the half peak oxidation potentials, other factors that are considered as being important for the prediction of the antioxidative potential of flavonoids, are the scavenging and decay rate constants and the pK values (Bors *et al.*, 1998). A recent comparison (Lemańska *et al.*, 2001) of the pKa values with the pH-dependent antioxidant profiles, has revealed that for various hydroxyflavones the pH-dependent behavior is related to hydroxyl moiety deprotonation, resulting in an increase of the antioxidant potential upon

formation of the deprotonated forms. Based on the results obtained it was then concluded that upon deprotonation the antioxidant activity value as determined by the $ABTS^{\bullet+}$ increases because electron-, not $H^{\bullet}$-donation becomes easier. Therefore the difference observed between the activity of the phenolic compounds obtained with the $ABTS^{\bullet+}$ assay as opposed to those of the FRAP assay could be partly attributed to the differing pH of the test mediums. The low pH of the FRAP assay would favor electron transfer of the compounds. The activity of formononetin in the FRAP assay (contrary to the lack of activity towards the $ABTS^{\bullet+}$) indicated the ability to donate electrons at a low pH (3.6). The lack of activity (Hubbe, 2000) exhibited by formononetin against the superoxide radical at a pH of 7.4 indicates that pH may play an important role in the antioxidant activity or electron donating ability of formononetin. However, it must be kept in mind that *in vivo* these compounds will exert their effects at physiological pH (pH = 7.4) so activity shown at a lower or higher pH is of little importance. Another factor concerning the lack of activity of formononetin in the $ABTS^{\bullet+}$ assay that should also be taken into consideration is the sterical hindrance between the radical cation and the phenolic compound. Similar to the results obtained in the present study Ruiz-Larrea *et al.* (1997) found formononetin to have a very low activity in the $ABTS^{\bullet+}$ assay. Steric hindrance between a polyphenol and a similar radical cation to the ABTS radical cation, also measuring hydrogen donating ability was proposed by Yoshida *et al.* (1989). He postulated that the degree of accessibility of the DPPH radical to the polyphenols would play a role in their apparent hydrogen donating ability. Formononetin has also been shown to lack hydrogen donating abilities towards this radical cation (Hubbe, 2000).

In the present study mangiferin exhibited the highest antioxidant activity in both the $ABTS^{\bullet+}$ and FRAP assays. The excellent $ABTS^{\bullet+}$ scavenging and ferric reducing activity of mangiferin can be contributed to the catecholic (6,7-dihydroxy) structure that is more susceptible to oxidation than the meta-di-substituted (1,3-dihydroxy) ring, since a radical formed at the 6-OH may be delocalised to the 7-OH and a radical formed at the 7-OH may be delocalised to 6-OH and the heterocyclic oxygen. In addition, radicals formed at the 1-OH, 3-OH and 6-OH positions in the xanthone structure may also be delocalised tot the carbonyl group (D. Ferreira, National Centre for Natural Product Research, University of Missippi, United States of America, personal communication, 1999, *as cited by* Hubbe, 2000).

Luteolin found to be more effective than mangiferin in other assays (i.e. hydrogen donating ability and $ABTS^{\bullet+}$ scavenging abilities as assessed by DPPH and superoxide radical scavenging) (Hubbe, 2000) contains two of the three structure activity requirements (Borse *et al.*, 1990) for antioxidant activity, namely the *ortho*-dihydroxy structure in the B ring and the 2,3-double bond in conjugation with the 4-oxo function in the C ring. These structural characteristics confer higher stability to the radical form and participate in electron delocalisation. The third requirement is the presence of the 3- and 5-OH groups with the 4-oxo functions in the A and C ring of which luteolin has one (5-OH group), for maximum scavenging potential. The structures of luteolin and eriodictyol differ only by the absence of the 2,3-double bond in the latter, resulting in an expected reduced $ABTS^{\bullet+}$ scavenging activity. According to Rice-Evans *et al.* (1996), flavanones having a saturated heterocyclic C-ring in contrast to the flavones and flavanols, have a lower H-donating ability as tested with the $ABTS^{\bullet+}$ assay, due to the lack of conjugation between the A and C ring. Contrary to the activity obtained for eriodictyol and luteolin with the $ABTS^{\bullet+}$ assay the ferric reducing activity of eriodictyol was far greater than that of luteolin. The absence of the 2,3-double bond therefore, did not decrease antioxidant activity in the FRAP assay, but enhanced it, suggesting greater electron donating ability.

The substitution of a *para*-OH group in the B ring of hesperetin by a methoxy group decreased its H-donating ability by *c.* 4 fold in comparison with that of eriodictyol. Hubbe (2000) found this decrease in H-donating ability as assessed by $DPPH^{\bullet}$ scavenging to be greater, at approximately 17 fold. This illustrates the importance of the presence of the 3',4'-dihydroxy configuration in the B ring. The methoxy group on the B ring of both hesperetin and its rhamnoside, hesperidin, is accountable for the loss of activity. The slightly higher activity obtained for the rhamnoside, hesperidin, was not expected since other studies have shown hesperetin with higher activity against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ (Rice-Evans *et al.*, 1996; Hubbe, 2000). However, hesperidin was shown to be more active that hesperetin in scavenging of the superoxide anion radical (Hubbe, 2000).

The importance of the 3', 4'-dihydroxy configuration can also be illustrated by the significant difference in antioxidant activity of naringenin versus eriodictyol. The activity of eriodictyol was 19 fold greater than that of naringenin in the $ABTS^{\bullet+}$ assay and 373 fold greater in the FRAP assay

In the lipophilic phase the specific mode of inhibitory action of the individual polyphenolic compounds is not clear, but they may act by chelating metal ions, preventing

chain initiation by scavenging initiating radicals or by scavenging lipid alkoxyl and peroxyl radicals thus acting as chain-breaking antioxidants (Terao & Piskula, 1998). It has generally been assumed that the ability of flavonoids to chelate Fe^{2+} is very important for their antioxidant activity, because "site specific scavenging" may occur (Haenen *et al.*, 1993). This means that if the Fe^{2+} is still catalytically active, the radicals are formed in the vicinity of the flavonoid, which surrounds the Fe^{2+}, and can be scavenged immediately. Thus the flavonoid would have a double, synergistic action, which would make it an extremely powerful antioxidant. Van Acker *et al.* (1996) stated that aglycones are more effective than glycosides in inhibiting lipid peroxidation, because of their extra chelation site, lower Ep/2 value and their increased lipophilicity. In the present study the pure compounds had lipid peroxidation inhibiting properties in the order of eriodictyol > luteolin > hesperetin > mangiferin > hesperidin > naringenin with formononetin ineffective. This order is in agreement with the previous statement regarding the Ep/2 values and the effect of glycosylation by Van Acker *et al.* (1996). However, the chelating abilities of these compounds does not seem to affect their ability to inhibit lipid peroxidation, since compounds shown to have no or very low chelating abilities such as hesperetin and hesperidin still performed better than compounds with good chelating abilities such as naringenin (Van Acker *et al.*, 1996). Van Acker *et al.* (1998) found that in microsomal lipid peroxidation, iron chelation did not appear to play a role for most of the flavonoids that they tested and only flavonoids with low $ABTS^{\bullet+}$ scavenging activities would benefit from their ability to chelate iron. Mangiferin with two chelating sites was much less effective at inhibiting Fe^{2+} induced lipid peroxidation, than as radical scavenger or Fe^{3+} reducer. According to Sato *et al.* (1992) mangiferin does not have the ability to scavenge peroxy radicals involved in lipid peroxidation, but that its activity is probably due to the ability to scavenge free radicals involved in the initiation of lipid peroxidation. Owing to the small role that iron chelation plays in inhibiting microsomal lipid peroxidation for the flavones and flavanones, other factors such as polarity and configuration should be considered. Compounds, with a more planar conformation such as the flavones in comparison with the tilted configuration of the flavanones are able to associate better with the membrane interface and thus are better equipped to inhibit or terminate lipid peroxidation in the propagation or termination reactions (Van Dijk *et al.*, 2000). In addition to this flavones are always slightly more hydrophobic than their corresponding flavanones giving these compounds a higher affinity for the lipophilic phase (Van Dijk *et al.*, 2000). In the present study however,

eriodictyol was slightly more effective than luteolin, indicating that the method was not effective in discriminating between compounds on this basis, or that other factors also play a role. It should be taken into consideration that flavonoids have also been shown to behave as prooxidants under certain conditions. These effects have been shown to be dependent upon the number of hydroxyl groups for flavones, isoflavones or flavanones (Cao *et al.*, 1997). In the present study naringenin with one less hydroxyl group than eriodictyol had a much lower protective effect against lipid peroxidation. Miranda *et al.* (2000) found that naringenin showed a tendency as prooxidant during exposure of LDL to Cu^{2+}. Naringenin has also been found to induce concentration-dependent peroxidation of membrane lipids in isolated rat liver nuclei (Sahu & Gray, 1997)

Antimutagenic activity against 2-AAF induced mutagenesis of the phenolic compounds tested on an equal molar basis (300 μM), showed only luteolin to have considerable antimutagenic activity (87%). This was in agreement with findings by Choi *et al.* (1994) who found luteolin to have good antimutagenic activity against aflatoxin B_1 induced mutagenesis in TA100 at a concentration of 388 μM. Naringenin at a concentration of 408 μM and hesperetin at 367 μM had good antimutagenic activity (85 and 88%, respectively) comparable to that of luteolin (Choi *et al.*, 1994). Under the test conditions used in the present study naringenin was highly promutagenic (-326%), while hesperetin had only a small antimutagenic effect (9%). The promutagenic effect of hesperidin (-9%) was also observed by Choi *et al.* (1996) against aflatoxin B_1 in TA100. However, compounds that exhibited promutagenic effects in the present study did not show mutagenic responses when tested at the same concentration in the presence and absence of S9 in TA98. The results obtained for hesperidin were in agreement with another study that tested for mutagenic responses with and without S9 in the frameshift strain TA98 (Hardigree & Epler, 1978). Mangiferin with very limited antimutagenic activity (8%) against 2-AAF in the present study did not exhibit any mutagenic activity in the tester strains TA97, TA98, TA100, and TA2637 with and without metabolic activation in a study by Matsushima *et al.* (1985). Compounds that are more lipophilic have been proven to exert greater antimutagenic effects than their corresponding glycosides. However, formononetin containing only a hydroxyl and a methoxyl group and thus of lipophilic character was the most promutagenic after naringenin. Very weak antimutagenic effects have been noted for other isoflavones genistein and biochanin A against N-hydroxy-IQ-2-amino-3-methylimidazo[4,5-f]quinoline in TA98 (Edenharder *et al.*, 1997). Biochanin A differs from

fomononetin by the presence of a 5-OH group. The effect of polarity on antimutagenic activity could however, be noted for hesperetin versus that of its glycoside, hesperidin and the more polar eriodictyol. Although hesperetin only exhibited weak antimutagenic activity, hesperidin and eriodictyol were both promutagenic. Furthermore, the major difference in the antimutagenic activity of luteolin versus eriodictyol that differs only by the 2,3-double bond suggests that this bond may be essential for activity.

Solvent extraction is generally used for isolation of antioxidants (Moure *et al.*, 2001). Both the extraction yield and antioxidant and antimutagenic activities of the solvent extracts were found to be strongly dependent on the polarity of the solvents used in this study. It was found that antioxidant activity of the extracts measured with the $ABTS^{\bullet+}$ and FRAP assays increased significantly with increasing solvent polarity. The reason for this was the improved extraction of total polyphenols with increasing solvent polarity. Contrary to this, the total polyphenol content was not important for inhibition of lipid peroxidation and antimutagenicity, as the non-polar character of the extracting solvent was the most important factor in determining activity. This was illustrated by the increased ability of the non-polar ethyl acetate extract in comparison to the methanol and water extracts to inhibit lipid peroxidation and mutagenesis. Although the polarity of the dichloromethane extract was lower than the ethyl acetate extract it is thought that most of the activity of the former is attributable to pheophytin a and b shown to be both strong antimutagens (Higashi-Okai *et al* 1997) and antioxidants (Higashi-Okai *et al.*, 2000) while that of the latter can be attributed to the presence of both these compounds and other lipophilic flavonoids resulting in a higher activity presumably due to their higher potency or higher quantities present. The difference in activity between sequential and non-sequential water extraction which was significantly lower with sequential extraction indicates that some compounds removed by the methanol extraction were able to be removed with water in the non-sequential extract. The effects of this could also be observed by the different intensities of the bands when these extracts were spotted at equal concentrations on TLC plates. The increased antimutagenic activity of the sequential methanol extract over the non-sequential extract indicated that antagonistic effects could have existed between compounds extracted with methanol and those not removed by ethyl acetate in the non-sequential extraction, especially since the compounds of the ethyl acetate extract were highly antimutagenic on their own. Although the ethyl acetate extract exhibited excellent protective properties against mutagenesis and lipid peroxidation it had only moderate $ABTS^{\bullet+}$ scavenging and ferric reducing activity as well as a low total

polyphenol content. The extract yield obtained from the ethyl acetate extract was also too small to be considered for further fractionation studies although further investigation may provide some very interesting data regarding antimutagenic activity of its compounds. The methanol extract that provided the greatest yield, total polyphenol content, $ABTS^{\bullet+}$ scavenging and ferric reducing activities was considered suitable for further fractionation. Although it had a lower antimutagenic activity than the ethyl acetate extract it was able to inhibit lipid peroxidation to some extent.

Fractionation of the methanol extract on XAD yielded fractions A-F in order of decreasing polarity and antioxidant activity and increasing antimutagenic activity. As for the solvent extracts total polyphenol content was important for activity measured in the $ABTS^{\bullet+}$ and FRAP assays but not in the ability to inhibit lipid peroxidation. Based on an additive effect with no synergism or antagonism the hesperidin concentration in fraction F had an apparent contribution of 30 and 64% towards the $ABTS^{\bullet+}$ scavenging and ferric reducing activities, respectively. Mangiferin + isomangiferin content could only account for 18-26% of the $ABTS^{\bullet+}$ scavenging and ferric reducing activity illustrating that although these compounds are the major compounds present in these fractions there may be other more active compounds or synergistic relationships that may exist. Synergistic actions between antioxidants have been observed (Hudson & Lewis, 1983; Frankel, 1996; Duh *et al.*, 1997; Heinonen *et al.*, 1997; Hattori *et al.*, 1998; Meyer *et al.*, 1998a; Meyer *et al.*, 1998b; Wijewickreme & Kitts, 1998; Bandarra *et al.*, 1999; Saucier & Waterhouse, 1999). This effect is defined as the combined action which results in increased antioxidant potential more than that expected from a mere additive effect. Meyer *et al.* (1998a) found interactive effects between flavonoids and phenolic acids. However the simultaneous presence of some compounds may present lower activity than expected; in this way antagonistic effects were observed between ellagic acid and catechin. Synergistic antioxidant effects between the compounds found in natural extracts are probably responsible for the higher antioxidant activity observed of the crude extracts than that measured in simulated extracts (Onyeneho & Hettiarachchy, 1992; Rodríguez de Sotillo *et al.*, 1994; Tsuda *et al.*, 1994).

Total polyphenol content could be shown to be a determining factor for the $ABTS^{\bullet+}$ scavenging and ferric reducing ability of the fractions while polarity and composition were of importance in inhibition of lipid peroxidation. Only very small quantities of the most effective compound at inhibiting lipid peroxidation tested in this study, the aglycone, eriodictyol, were present in fraction E and F, yet these fractions were of comparable activity

to fraction D, which contained twice as much eriodictyol. Therefore this observed protective effect of fraction D can be attributed to the major compounds mangiferin + isomangiferin, which was moderately effective while hesperidin, which had low efficacy but was present in large quantities, was responsible for the protective effect of fraction E and F against lipid peroxidation. The antimutagenic effect of fraction F was not affected by the high concentration of hesperidin (39% of the fraction and 78% of the total polyphenol content), shown to have a promutagenic effect on its own. Therefore the activity of fraction F could either be accounted for by the high activity of the remaining 60% of the fraction and 12% of the total polyphenols or due to synergistic relationships between the compounds not accounted for in the present study. Not taking into account fraction A that exhibited very weak antioxidant and antimutagenic activity fraction E provided not only the highest extract yield but also moderate antioxidant activity and excellent antimutagenic activity. Based on these factors fraction E was considered as the most favorable fraction for further fractionation.

Reversed-phase fractionation of fraction E yielded fractions E_1-E_6 in order of decreasing polarity. Fractions that exhibited high $ABTS^{\bullet+}$ scavenging and ferric reducing activity had correspondingly higher total polyphenol contents. The ability of the fractions to inhibit lipid peroxidation did not differ to a large extent from fraction to fraction (69-73% inhibition) while the antimutagenic activity did. The differences in antimutagenic activity of the fractions were not in order of decreasing polarity as for the XAD fractions, with fraction E_6 followed by E_1 exhibiting the highest antimutagenic abilities. Both these fractions did not contain any of the compounds tested. However, the presence of polyphenols was indicated by the reaction with the Folin-Ciocalteu reagent. This suggested the presence of compounds with high antimutagenic activities or synergistic effects since fraction E_6 had the lowest total polyphenol content (11%). Fraction E_6 was shown to contain most of the potent antimutagen, luteolin (1.15%), and hesperetin (0.06%) of less potency which could account for some of the activity observed. Fraction E_1 containing the more polar compounds of lesser efficacy, had the highest total polyphenol content. Both of these fractions merit further investigation so that their composition so that synergistic and/or antagonistic effects of the major constituents can be elucidated.

CONCLUSIONS

This study has shown that there are significant differences in antioxidant and antimutagenic activities of different phenolic compounds present in *C. intermedia* and that their contribution to the total activity of extracts or fractions cannot necessarily be quantified from the sum of their individual activities, due to synergistic or antagonistic effects that may occur between compounds. More polar mixtures exhibited better antioxidant activity in the $ABTS^{\bullet+}$ and FRAP assays than the non-polar mixtures that provided better protection against microsomal lipid peroxidation and 2-AAF induced mutagenesis. While $ABTS^{\bullet+}$ scavenging and ferric reducing activities of extracts and fractions could be predicted on the basis of their total polyphenol content, their ability to inhibit lipid peroxidation and mutagenesis could not.

In order to obtain a better understanding of the bio-activities of fractions from unfermented *C. intermedia* more information on their phenolic composition, the performance of these compounds in other antioxidant assays and the mechanism involved in the inhibition of mutagenesis is required. The characterisation and quantification of the phenolic compounds and/or other unknown compounds in these fractions will provide more information regarding the synergistic and/or antagonistic effects noticed in the different fractions. It will also provide important information about the chemical characteristics of the major antimutagenic compounds and their relation to the antioxidant properties of honeybush tea.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Although tea cannot be granted official health claims at present, it can be recognised as an important part of a sound diet. Moving from a traditional beverage associated with rituals, tea is now viewed as a healthy drink, a source of pharmacologically active molecules, an important member of the antioxidant food group, and a functional food endowed with beneficial health properties (Dufresne & Farnworth, 2001). New products and uses are emerging and tea is consumed in different manners. Iced tea is now a convenient alternative to soft drinks and is found in grocery stores and fast food outlets. Recently encapsulated green tea (*Camellia sinensis*) and green rooibos tea (*Aspalathus linearis*) extracts appeared on the health store shelves for those who do not like to drink these teas, but who seek their benefits.

The antioxidant and antimutagenic properties of *C. sinensis* teas have been well studied (Zhao *et al.*, 1989; Salah *et al.*, 1995; Yen & Chen, 1995; Najo *et al.*, 1996; Van Acker *et al.*, 1996) and reviewed (Namiki, 1990; Graham, 1992; Yang & Wang, 1993; Dreosti, 1996; Weisburger, 1996), lending credibility to health claims that are made. On the other hand, little information is available on the health promoting properties of *Cyclopia* species used to prepare honeybush tea, with only two studies on the antioxidant (Hubbe, 2000) and antimutagenic (Marnewick *et al.*, 2000) activities of some of the *Cyclopia* species conducted up to date. Hubbe (2000) found that fermentation lowered their antiradical efficiency towards DPPH radical and superoxide anion radical, while the ability to inhibit linoleic acid peroxidation was not affected by fermentation. Unfermented *C. sessiliflora*, of the species tested, *C. intermedia*, *C. genistoides*, *C. subternata*, *C. sessiliflora* and *C. maculata*, had the highest total polyphenol content and was the most effective at scavenging the DPPH and superoxide anion radicals, while *C. genistoides* showed the most protection against linoleic acid peroxidation. The ability of *C. intermedia* to inhibit mutagenesis induced by 2-AAF and aflatoxin B_1 in TA98 and TA100, shed more light on the modes of antimutagenic action (Marnewick *et al.*, 2000). The strong antimutagenic effect exhibited by unfermented *C. intermedia* towards 2-AAF and AFB_1 was thought to be due to the polyphenols either acting as electron acceptors to NADPH, impeding the flow of electrons to cytochrome P_{450} and preventing the formation of genotoxic intermediates or through a direct

interaction with the activated mutagenic metabolites or the different components of the enzyme system catalysing the metabolic activation process.

In the present study the antioxidant and antimutagenic activities of four species of *Cyclopia* with economic potential (E. Joubert, ARC Infruitec-Nietvoorbij, personal communication, 2002), namely *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides*, were compared. The aqueous extracts of the unfermented species had significantly higher $ABTS^{\bullet+}$ scavenging and ferric reducing activities than their fermented counterparts. Unfermented *C. intermedia* had the highest $ABTS^{\bullet+}$ scavenging and ferric reducing activity. In comparison with other commercial brands of tea the ferric reducing activity of the aqueous extracts of *Cyclopia* and more specifically that of unfermented *C. intermedia* (11.22 μmol mg^{-1}) are comparable to that of black tea (11.71 μmol mg^{-1}) and unfermented rooibos (11.31 μmol mg^{-1}), but not to green tea (*Camellia sinensis*) (14.65 μmol mg^{-1}) (Richards *et al.*, 2001). The much higher antioxidant activity of the latter is mostly attributable to its high flavanol content (14.26%), consisting primarily of the potent antioxidants (Rice-Evans *et al.*, 1996), the catechins and gallocatechins (Wiseman *et al.*, 1997). In comparison to the aqueous extracts of green tea, *C. intermedia* only contained a small percentage of flavanols (2.34%). At this stage it is not clear whether the reaction given with the DAC reagent was due to the presence of monomeric and/or polyhydroxyflavans. Catechins and gallocatechins are absent from fermented *C. intermedia* (Ferreira *et al.*, 1998; Kamara, 1999).

Unfermented species gave better protection against lipid peroxidation in a rat liver microsomal system than their fermented counterparts. Unfermented *C. sessiliflora* gave the highest protection and *C. genistoides* the least. The ability of *C. genistoides* to inhibit lipid peroxidation did not change with fermentation and it was the most potent of the fermented species. The protective effect of the other species decreased with fermentation and their activity was not significantly different from each other.

Fermentation also affected the protective effect of the most potent species, *C. intermedia* and *C. sessiliflora*, against 2-AAF induced mutagenesis. In both cases their antimutagenicity was lowered substantially. Fermentation had no effect on the antimutagenicity of *C. subternata*, and this species, together with *C. sessiliflora*, were the most potent of the fermented teas. Fermentation caused *C. genistoides* to change from being promutagenic to antimutagenic. However, *C. genistoides* was not mutagenic in the absence

and presence of metabolic activation. Fermented *C. intermedia* and *C. genistoides* displayed the least amount of antimutagenic activity.

Based on the overall performance of unfermented *C. intermedia* as antioxidant and antimutagen and its economic importance, as well as the extent of its commercial exploitation and the availability of plant material and information on the phenolic composition, further investigation of this species through selective extraction with solvents of different polarities and the use of activity-guided fractionation, was carried out.

Differences in phenolic composition could explain the differences in activity obtained for the different species, as well as for the unfermented and fermented plant material. A large difference in the phenolic composition shown between fermented *C. intermedia* (Ferreira *et al.*, 1998; Kamara, 1999) and unfermented *C. subternata* (Brand, 2002) indicated that there may be further differences among the other species of *Cyclopia* or that fermentation could have an effect on the phenolic composition. Based on HPLC analysis, the present study showed that qualitative and quantitative differences existed between the species investigated. Major peaks that could not be identified as being mangiferin, hesperidin, naringenin, eriodictyol, hesperetin, luteolin or formononetin were present in the aqueous extracts of unfermented *C. subternata* and *C. intermedia.* These peaks were shown to decrease substantially with fermentation, indicating the potential of the compounds as antioxidants due to their susceptibility to oxidation. The unidentified peak in the aqueous extract of unfermented *C. intermedia* was also present in HPLC chromatograms of the less polar XAD fractions, E and F, and the C_{18} fractions, E_5 and E_6. These fractions exhibited moderate antioxidant and antimutagenic activities. Identification of the compound could provide further insight into the antioxidant and antimutagenic activities of the aqueous extracts and fractions, especially since this compound seems to be present in much larger quantities in *C. intermedia* than the other *Cyclopia* species.

The antioxidant and antimutagenic activities of phenolic compounds present in unfermented *C. intermedia* provided more insight into their apparent contribution to the antioxidant and antimutagenic potential of the extracts and column fractions. Of the compounds tested, mangiferin had the highest $ABTS^{\bullet+}$ scavenging and ferric reducing abilities and a moderate ability to inhibit lipid peroxidation. However, almost no protection against 2-AAF induced mutagenesis was observed for this polar compound. Mangiferin + isomangiferin, together with hesperidin, were identified as the major compounds of all of the *Cyclopia* species investigated in this study. This is in agreement with results obtained by

Joubert *et al.* (2002) on unfermented *C. intermedia*, *C. sessiliflora* and *C. genistoides*. Most of the antioxidant activity of the species could however, not be accounted for by the major compounds mangiferin + isomangiferin and hesperidin. Unfermented *C. genistoides*, containing large amounts of mangiferin + isomangiferin (12%), did not exhibit higher activity in the $ABTS^{\bullet+}$ and FRAP assays than unfermented *C. intermedia* with only 3% mangiferin + isomangiferin. This indicated possible synergistic relationships between the compounds of the mixture, the presence of very potent trace compounds and/or the contribution of polymeric compounds such as proanthocyanidins. The presence of the latter compounds was indicated by the reaction given by the aqueous extract with 4-dimethylaminocinnamaldehyde, since fermented *C. intermedia* contains no monomeric catechins or gallocatechins (Ferreira *et al.*, 1998; Kamara, 2000).

The antimutagenic and the sometimes promutagenic activity of some of the pure compounds tested could have profound effects on the overall antimutagenic potential of the extracts or column fractions. The enhancement of 2-AAF induced mutagenesis by naringenin, eriodictyol and formononetin, and to a lesser extent, hesperidin, could partly explain the difference in the antimutagenic activity of *C. intermedia*, *C. subternata* and *C. sessiliflora*, as well as the promutagenic activity of unfermented *C. genistoides*. Naringenin, eriodictyol and formononetin were not present or only present in very low quantities in the extracts. The hesperidin content was greatest in the aqueous extract of unfermented *C. intermedia* (1.3%) followed by unfermented *C. genistoides* (1.1%), yet unfermented *C. intermedia* was not promutagenic. Although *C. genistoides* contained slightly less hesperidin than *C. intermedia*, its flavonol + flavone content was substantially higher than that of *C. intermedia* (8.5 versus 3.3%). It was shown that the flavonol + flavone content had a highly significant negative correlation with antimutagenic activity ($r = -0.60$; $P < 0.0001$). The change in activity of *C. genistoides* from promutagenic to antimutagenic with fermentation suggested that oxidation of the compounds resulted in an antimutagenic response, and/or that their lower concentration after fermentation, resulted in the nett antimutagenic activity. This scenario presents a problem when using the *Salmonella typhimurium* assay to asses extracts for antimutagenic activity, because the presence of compounds that may enhance the mutagenicity of 2-AAF will result in an underestimation of activity. The use of a variety of mutagens and various tester strains should give a better evaluation of the antimutagenic potential of *Cyclopia* since different mechanisms and interactions are involved. These interactions could be between the phenolic compounds and the mutagen, promutagen or

activated metabolites or with the enzyme system involved in the metabolic activation of the mutagen.

In the present study total polyphenol content was shown to be consistently correlated ($r \geq 0.97$; $P < 0.05$) with antioxidant activity measured in the $ABTS^{\bullet+}$ and FRAP assays. This was in agreement with studies on various other plant extracts (Frankel *et al.*, 1995; Meyer *et al.*, 1997; Benzie & Szeto, 1999; Deighton *et al.*, 2000; Gardner *et al.*, 2000; Luximon-Ramma *et al.*, 2002). Antioxidant activity against rat liver microsomal lipid peroxidation and antimutagenic activity towards metabolically activated 2-AAF could not always be correlated with the total polyphenol content. For instance the total polyphenol content of aqueous extracts of unfermented and fermented *Cyclopia* species was well correlated with their ability to inhibit microsomal lipid peroxidation ($r = 0.79$; $P < 0.0001$), but not with their ability to inhibit mutagenesis induced by metabolically activated 2-AAF ($r = -0.082$; $P = 0.5630$). Contrary to this, solvent extracts of unfermented *C. intermedia* showed a good correlation between their total polyphenol content and antimutagenic activity against 2-AAF ($r = 0.84$; $P < 0.0001$), while no correlation between total polyphenol content and the ability to inhibit lipid peroxidation ($r = -0.3$; $P = 0.2231$) was found. The opposite effect was again illustrated for the fractions from a methanol extract of unfermented *C. intermedia* where the crude fractions illustrated a good correlation between total polypenol content and ability to inhibit lipid peroxidation ($r = 0.70$; $P < 0.05$). This correlation was lost upon further fractionation. Other studies (Marnewick *et al.*, 2000; Yen & Chen, 1995) also found conflicting results with regard to the correlation between total polyphenol content and antimutagenic activity, especially when using different mutagens and tester strains. The consistent correlation of total polyphenol content with activity obtained with the $ABTS^{\bullet+}$ and FRAP assays, as opposed to the inconsistent correlations obtained for total polyphenol content with the ability to inhibit microsomal lipid peroxidation and 2-AAF induced mutagenesis, indicated the importance of phenolic composition as opposed to total polyphenol content. Other factors to be taken into consideration are the differing polarities of the extracts and fractions in relation to one another, as well as differences in the conformation and level of protonation of the compounds present (Edenharder & Tang, 1997; Marinova & Yonaishlieva, 1997; Van Dijk *et al.*, 2000; Lemańska *et al.*, 2001). This is notwithstanding the possible disruption of synergistic relationships between compounds upon solvent extraction and fractionation (Moure *et al.*, 2001).

In the present study antimutagenic activity could be linked to the polarity of the extract when considering the solvent extracts of unfermented *C. intermedia*. The ethyl acetate extract (87% inhibition) had greater antimutagenic activity than the more polar non-sequential methanol extract (9% inhibition). Removal of the ethyl acetate soluble compounds before methanol extraction resulted in a higher antimutagenic activity (32%), indicating a possible antagonistic effect of these compounds in the non-sequential methanol extract. Polarity also affected the antimutagenic activity of the XAD fractions, A to F, with fraction A being the most polar and slightly promutagenic (-10%) to fraction F, the least polar and highly antimutagenic (85%). However, this relationship broke down with further fractionation of fraction E on C_{18}. The C_{18} fractions did not follow the same trend with polarity, suggesting the contribution of individual compounds, rather than the nett effect of a group of compounds. The effect of polarity on antimutagenic activity observed for the solvent extracts and XAD fractions was also found by Edenharder *et al.* (1997).

A definite correlation between the antioxidant activity determined with the $ABTS^{\bullet+}$ and FRAP assays could be found throughout the entire study (i.e. for aqueous extracts of different *Cyclopia* species and solvent extracts and fractions of unfermented *C. intermedia*) ($r \geq 0.98$, $P < 0.0001$). This correlation was demonstrated in another study on rosehip extracts (Gao *et al.*, 2000). Concerns regarding the validity of the FRAP assay as a measure of antioxidant activity due to its pro-oxidant character have been raised (Prior & Cao, 1999). The fact that this assay measures the ability of compounds to reduce Fe^{3+} to Fe^{2+} raises concerns due to the ability of Fe^{2+} to take part in the Fenton reaction leading to the generation of hydroxyl radicals *in vivo* (Halliwell & Gutteridge, 1989). The potential for metal ion induced oxidation *in vivo* is a controversial topic, since iron must be "free" to be catalytically active, and not stored in proteins like hemoglobin, myoglobin, ferritin, hemosiderin or transferrin (Sergent *et al.*, 1999). However, even though these proteins scavenge divalent ions in plasma, advanced arteriosclerotic lesions have been shown to contain significant amounts of catalytically active copper and iron (Witztum & Steinberg, 2001). Although the FRAP assay may not be used to predict antioxidant activity relative to an *in vivo* situation, it is a very useful alternative method that can be used for rapid screening of extracts. Slight differences were observed between activity determined using the $ABTS^{\bullet+}$ assay as opposed to the FRAP assay when the pure compounds, mangiferin, luteolin, hesperetin, hesperidin, naringenin, eriodictyol and formononetin, were tested. This difference could be attributed to various effects such as structural hindrance between the phenolic compound and the synthetic

ABTS radical cation [similar to that exhibited by the DPPH radical (Yoshida *et al.*, 1989)] or due to the effect of pH. Lemańska *et al.* (2001) have shown that pH has an effect on the antioxidant activity profile of hydroxyflavones. This would explain differences in antioxidant activity determined with the FRAP assay, carried out under acidic conditions in comparison with activity determined at the neutral pH of the $ABTS^{\bullet+}$ assay. While assays such as the FRAP assay (not carried out at physiological pH) remain useful tools for rapid screening of extracts or fractions, methods carried out at physiological pH such as the microsomal lipid peroxidation assays would be of greater significance for extrapolations with *in vivo* data.

The present study has confirmed the potential of honeybush as a nutraceutical or functional beverage and has laid the foundation upon which additional and more extensive *in vitro*, as well as *in vivo* studies can be based. Several of the aqueous extracts of the different *Cyclopia* species and the solvent extracts and column fractions of unfermented *C. intermedia* have exhibited both antioxidant and antimutagenic activities, meriting further comprehensive investigations. Fractionation has provided useful information on the distribution of activity, especially with regard to the polarity of the column fractions. This is of special importance if fractions are to be selectively used for the production of nutraceutical products with specific biological activities. Furthermore, future studies should aim to provide more information on the phenolic composition of these fractions, which will help to explain possible synergistic and/or antagonistic relationships that may exist.

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ADDENDUM A

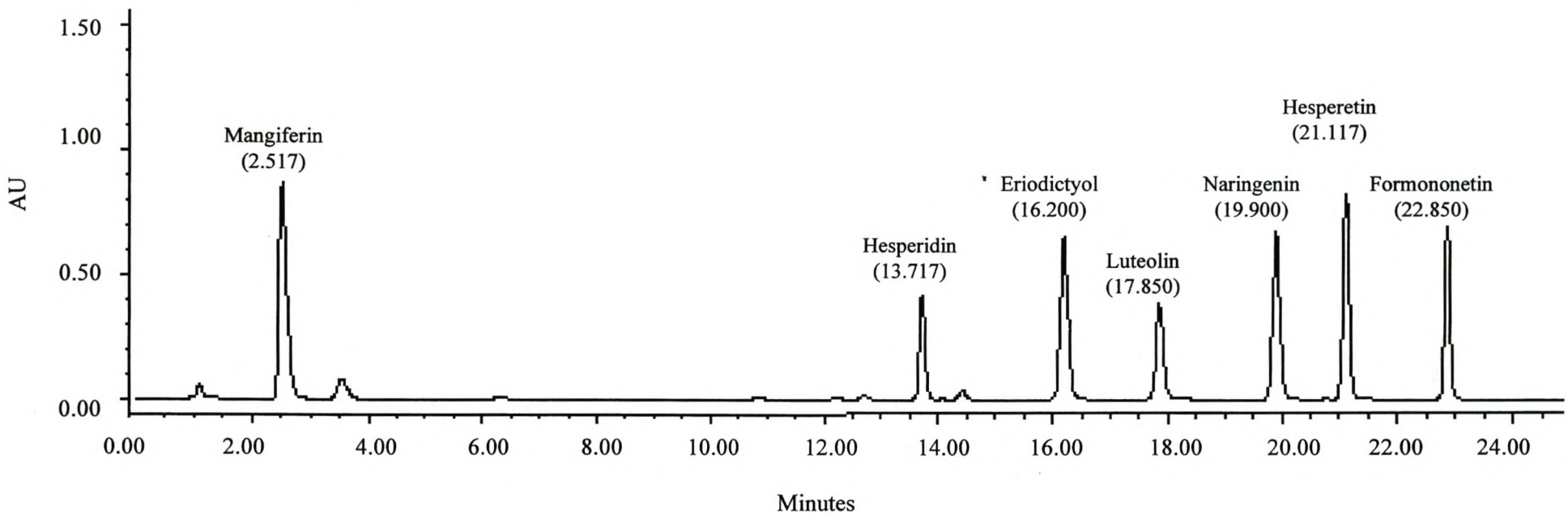

Figure 1. Chromatogram with the retention times (minutes) in brackets of the pure compounds mangiferin, hesperidin, eriodictyol, luteolin, naringenin, hesperetin and formononetin. HPLC analysis was carried out on a Zorbax SB-C_{18} (3.5 µm particle size, 150 x 3 mm) column under gradient conditions; mobile phase, acetic acid:acetonitrile; flow rate, 1 mL min^{-1}; detection, 280 nm; column temperature, 30°C.